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Comparative Biochemistry of Hemoglobins

VIII. Isolation of What is Called Soluble Tryptic Peptides from the β-Polypeptide Chain of *Macaca mulatta* Monkey Hemoglobin and their Amino Acid Compositions

Hiroshi TAKEI*

Department of Biochemistry,
Nagasaki University School of Medicine
Nagasaki, Japan

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By countercurrent distribution method with the system of water, sec.-butanol containing 0.08% trichloroacetic acid, and propionic acid (11.0 : 8.7 : 1.5), β-polypeptide chain of *Macaca mulatta* monkey hemoglobin was isolated. It was digested with trypsin. The peptides and an amino acid, which were contained in the part soluble at pH 6.4 of this hydrolysate, were isolated and refined by column and paper chromatography; column chromatography was carried out with Dowex 1×2 as the adsorbent, and with the buffer containing organic bases such as pyridine and collidine as the developer; paper chromatography, with the system of n-butanol, acetic acid, and water (4 : 1 : 5). Finally, the amino acid composition of these peptides and an amino acid were examined. As the result, the amino acid compositions of eleven peptides and one amino acid in, what is called, the soluble tryptic peptides of β-polypeptide chain of *Macaca mulatta* monkey hemoglobin were known. The comparison of amino acid composition of β-polypeptide chains between human and *Macaca mulatta* monkey hemoglobin demonstrated that there were six exchanges of amino acid compositions in five peptides. From this fact, it is presumed on the partial primary structure that β-polypeptide chain of *Macaca mulatta* monkey hemoglobin is different at least at six points in its amino acid sequence from that of human hemoglobin.

INTRODUCTION

In connection with the problem of molecular evolution of hemoglobin, the comparative biochemical studies on hemoglobins of various living matters are having been made in our laboratory. As hemoglobin

* 武居　洋

is a chromoprotein which is widely distributed in nature and as it can be comparatively easily isolated and refined, it has been studied well. Moreover, it is known that every hemoglobin has its own alkali resistance, its own rate of electrophoresis, and some other characteristics, and that this is not due to the protoheme of the hemoglobin but due to its globin part. The difference of the primary structure of the globin part of various hemoglobins is considered to be an accumulation of what is called point mutations caused by one change in amino acid sequence of globin which originated in the single base change of the gene that controls the biosynthesis of hemoglobin through the evolutional process of living matters. From this point of view, the determination of the primary structure of various hemoglobins will much contribute to the elucidation of the relation between the morphological evolution of these living matters and the molecular evolution of proteins which compose them.

Concerning the primary structure of hemoglobins, whole structure of human adult[2),9)] and fetal[20)] hemoglobin and the greater part of horse hemoglobin[12),22)] have been already known. Horse hemoglobin has 141 amino acids in α-polypeptide chain and 146 amino acids in ß-polypeptide chain just like human hemoglobin. However, concerning amino acid sequence, 17 exchanges in α-polypeptide chain and 26 exchanges in ß-polypeptide chain are recognized between these two hemoglobins. It was also reported that the amino acid sequence of gorilla hemoglobin was different from that of human hemoglobin at two points in α-polypeptide chain and at one point in ß-polypeptide chain.[28)]

On *Macaca mulatta* monkey hemoglobin, since SCHAPIRA and KRUCH[19)] recognized its immunological resemblance to human hemoglobin, CABANNES and SERAIN[3)] and RODNAN and EBAUGH[17)] by paper chromatography, and FINE et al[5)]. by agar gel electrophoresis confirmed the resemblance between these two hemoglobins. On the other hand, ZUCKERKANDL et al.[27)] reported that there were but a few differences between the two, comparing their what is called soluble tryptic peptides by fingerprinting method. However, MÄSIAR[11)] did not recognize the difference by comparing their soluble tryptic peptides. SHIKAYA[21)] compared the two hemoglobins by agar gel electrophoresis, CM-cellulose column chromatography, alkali denaturation method, and fingerprinting method. He reported that though they closely resembled each other, there were some clear differences on their primary structure. Furthermore, MAITA[10)] isolated and refined what is called soluble tryptic peptides of α-polypeptide chain of *Macaca mulatta* monkey hemoglobin by column chromatography using Dowex 1 × 2 and by paper chromatography, and analyzed their amino acid compositions. Comparing his results with those of human hemoylobin, he recognized five amino acid exchanges in three peptides between the two hemoglobins.

The present author isolated ß-polypeptide chain of *Macaca mulatta*

monkey hemoglobin by countercurrent distribution method. It was digested with trypsin. The soluble part at pH 6.4 in the hydrolysate was isolated and refined by column chromatography by using Dowex 1×2 as the adsorbent and the buffer containing organic bases as the developer, and by paper chromatography with the system of n-butanol, acetic acid, and water (4 : 1 : 5). Furthermore, the amino acid compositions were examined and compared with those of human hemoglobin.

MATERIALS AND METHODS

1) *Preparation of the ß-polypeptide Chain from Macaca mulatta Monkey Hemoglobin*

According to DRABKIN'S method[4], hemoglobin was separated from the blood punctured from the abdominal aorta of *Macaca mulatta* monkey and was changed into cyanmethemoglobin with potassium ferricyanide. It was dehemed by TEALE'S methyl ethyl ketone method[25]. According to MATSUDA et al.'s method[15], globin was separated into α and β-polypeptide chain by countercurrent distribution method with the system of water, sec-butanol containing 0.08 % trichloroacetic acid, propionic acid (11.0 : 8.7 : 1.5); that is, globin (0.5−1.0g) was dissolved into 50 ml of the lower phase of the above-mentioned system and was moreover added 50 ml of the upper phase. It was mixed and poured into Tube No. 3−7 of automatic countercurrent distribution apparatus (Shibata CO. CDA−100) which has one hundred 20 ml-tubes. They were shifted 150 times. The upper and lower phase of each tube were taken respectively. After they were dropped 50 % ethanol in order to remove turbidity, their extinction at 280 mμ were examined. The parts of upper and lower phase which contained α and β-polypeptide chain were gathered respectively, dialyzed against deionized water, and then lyophilized. To confirm the purity of the polypeptide chains obtained in this way, the analysis of N-terminal amino acid sequence was carried out by DNP-method; that is, 100 mg of polypeptide chain was dissolved into 20 ml of deionized water and adjusted to pH 9.0 with 0.1 N NaOH. It was added 25 mg of Na_2CO_3 and 0.05 ml of 2.4-dinitrofluorobenzene (DNFB) and dinitrophenylated for 2 hrs at 40°C. After this reaction, the pH was adjusted to 2.0. Consequently, yellow DNP-polypeptide chain precipitated. This precipitate was separated by centrifugation, washed with 0.1 N HCl, acetone, ether in order. Thereafter, it was dried in the desiccator in the dark room. DNP-α-polypeptide chain and DNP-β-polypeptide chain were hydrolyzed with 6 N HCl under reflux condenser for one hour. DNP-amino acid and DNP-peptides contained in the hydrolysate were extracted with ether and ethyl acetate. They were isolated, identified, and determined quantitatively by column chromatography by using silica-gel celite as the adsorbent and various kinds

of organic solvents.[13)14)]

2) *Digestion of the β-Polypeptide Chain with Trypsin and Isolation of What is Called Tryptic Peptides*

The hydrolysis of β-polypeptide chain with trypsin was carried out in the same way with the hydrolysis of α-polypeptide chain; that is, into 50 ml of 8 M urea solution, 500 mg of β-polypeptide chain was dissolved. After it was heated and denatured at 60°C for 45 min, it was cooled and dialyzed against deionized water to remove urea. By REDFIELD and ANFINSEN's method[16)], trypsin (Worthington Biochemical Co., twice crystallized) was dissolved into 1/16 N HCl to a concentration of 1 % and incubated at 37°C for 12 hrs in order to remove its chymotrypsin-like activity. The β-polypeptide chain denatured in urea solution precipitated in the dialyzing tube as the concentration of urea became lower by dialysis. The suspension of β-polypeptide chain was transfused into the incubator kept at 37°C and adjusted to pH 8.5 by adding 0.1 N NaOH. It was added 1 ml of trypsin solution prepared previously and digested. During the course of digestion, the pH of the suspension was kept at 8.0 by adding 0.1 N NaOH. On this occasion, the increase of the alkali uptake almost stopped in about 2 hrs, however, the solution was pretty cloudy. So another 0.5 ml of trypsin solution was added and the digestion was proceeded for 2 hrs. As the reaction mixture was still cloudy, it was subjected to the centrifugation (5000 r. p. m., 20 min) to remove the clouds. The hydrolysate obtained in this way was adjusted to pH 6.4 with 1 % acetic acid. Consequently, what is called "core" precipitated. After this hydrolysate was allowed to stand at pH 6.4 in the cold room overnight, it was separated by centrifugation (3000 r. p. m., 20 min). In this way, what is called soluble tryptic peptides from the supernatant solution of this hydrolysate. They were evaporated under reduced pressure and then lyophilized.

3) *Column Chromatography of the Soluble Tryptic Peptides on Dowex 1×2*

Dowex 1×2 (Dow chemical company, 200−400 mesh) was suspended in three volumes of deionized water, stirred, and allowed to stand for 20 min. Thereafter, it was removed very fine particles by the method of decantation and washed with 1 N NH_4OH, water, acetic acid, water. This resin (acetic acid type) activated in this way was suspended with three volumes of the starting buffer and evacuated thoroughly. It was loaded to the column (2×150 cm) which was kept at 37°C. After the resin in the column was thoroughly equilibrated with the starting buffer, the sample which was dissolved 40 ml of deionized water and adjusted to pH 9.0 with 0.1 N NaOH was put on the resin. Fraction No. 1 to No. 25 were developed with the starting buffer (1 % pyridiene − 1 % collidine-acetate buffer, pH 8.5). After Fraction No. 25, gradient elution was employed; that is, from Fraction No. 26 to No. 180, 0.075 N acetic

acid was poured to 1.5 L of 1% pyridine – 1% lutidine – 1% picoline acetate buffer pH 7.5. From Fraction No. 181 to No. 250, 1.0 N acetic acid was poured continously. Finally, the column was washed with acetic acid after Fraction No. 251. The effluent was collected in 20 ml fractions. The flow rate was 100 ml/hr. After alkali hydrolysis, the portion of every fraction was subjected to ninhydrin reaction according to YEMM and COCKING's method[26] and peptides were detected. Namely, from every fraction, 0.2 ml of effluent was taken in a hard-glass tube respectively and added 1 ml of 2.5 N NaOH. It was capped with aluminum foil. After hydrolyzed in the oil-bath for 2.5 hrs at 90°C, it was cooled and neutralized by adding 1 ml of 30 % acetic acid. From each tube, 1 ml of solution was taken respectively and added 0.5 ml of 0.2 M citrate buffer (pH 5.0) and 1.2 ml of ninhydrin-KCN solution. Every tube was again capped with aluminum foil and heated to color in the oil-bath at 100°C for 15 min. After that, it was cooled in the flowing water and diluted with 3 ml of 60% ethanol. Each extinction rate at 570 mμ was measured. The effluent of each ninhydrin-positive peak was collected and evaporated under the reduced pressure. It was dissolved into 3.0 ml of warm deionized water, stored in the freezer, and used in the next experiment.

4) *Paper Chromatography for Purification of Peptides*

Paper chromatography was performed by the descending method, spotting 0.03 ml from 3 ml of each sample, which was obtained by column chromatography mentioned previously. Toyo filter paper No. 50 (40×40 cm) and the developer whose system was n-butanol, acetic acid, and water (4 : 1 : 5) were used. Peptides on the paper were sprayed 0.2 % ninhydrin-butanol solution, heated with iron to color, and consequently detected. SAKAGUCHI's reaction,[8] PAULY's reaction,[24] α-nitrosonaphthol reaction,[1] and EHRLICH's reaction[23] were employed in order to confirm the peptides containing the amino acid which presents the specific reaction.

Paper chromatography for purifying peptides was performed with the same system described above, but for the ninhydrin color reaction 0.02 % ninhydrin-butanol solution was used. Ninhydrin-positive spots containing peptides were cut out, washed with acetone to remove ninhydrin, and dried in air. In the closed vessel, this cut-out paper, which was attached at the tip of the long filter paper strip and hung down from the petri dish filled with 5% acetic acid solution, was eluted into the beaker which was put below the petri dish. Finally, it was concentrated to dryness in the reduced pressure.

5) *Amino Acid Analysis of the Peptides*

Peptides purified by paper chromatography was dissolved into 4 ml of constant boiling point HCl (twice distilled) and hydrolyzed at 105°C

for 20 hrs or 40 hrs in a sealed tube for hydrolysis. The hydrolysate was concentrated to dryness and added small portion of deionized water. It was again concentrated to dryness to remove as much HCl as possible. The analysis of amino acids was performed by Hitachi KLA −2 amino acid analyzer by using 50 cm column and 7.5 cm column. The confirmation of tryptophan was carried out according to EHRLICH's reaction which peptides on the paper chromatogram showed.

RESULTS AND DISCUSSION

Fig. 1 shows the result of isolation of α and β-polypeptide chain from 0.5 g of *Macaca mulatta* monkey globin by countercurrent distribution method.

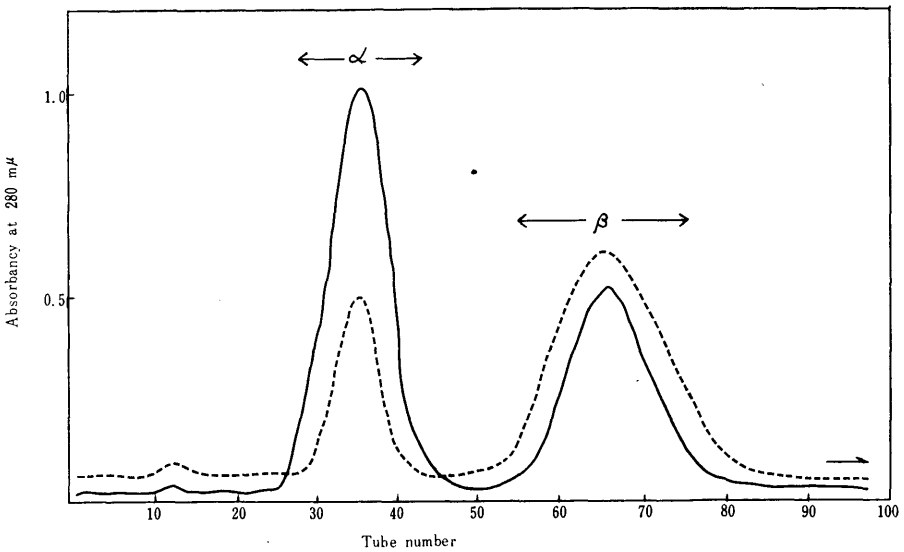

Fig. 1. Countercurrent distribution of the globin from *Macaca mulatta* monkey.
150 transfers, 10 cc in each phase. The sample was initially placed in tubes No. 3 − 7,.———. lower phase,.·····. upper phase

The analysis of N-terminal structure by DNP method was performed in order to examine the purity of α and β-polypeptide chain and the result was as follows. The values of DNP-Val, DNP-Val- Leu, DNP-Val-His, which were got by hydrolysis of DNP-α-polypeptide chain with 6 N HCl for one hour, were 0.58 mol, 2.63 mol; and 0.12 mol respectively per 1 mol of α-polypeptide chain regarding its molecular weight as 33,000. DNP-Val-His-Leu could not be recognized. On the other hand, in case of DNP-β-polypeptide chain, the values of DNP-Val, DNP-Val-Leu, DNP-Val-His, and DNP-Val-His-Leu were 0.55 mol, 0.10 mol,

1.61 mol, and 0.93 mol respectively. From this result, the purity of isolated α-and β-polypeptide chain may be at least more than 95 %. This result also accords with MATSUDA and MAITA's report[13] that N-terminal of *Macaca mulatta* monkey hemoglobin consists of 2 mols of Val-Leu and 2 mols of Val-His-Leu.

The β-polypeptide chain isolated and refined in this way was digested with trypsin. Alkali uptake curve was shown in Fig. 2.

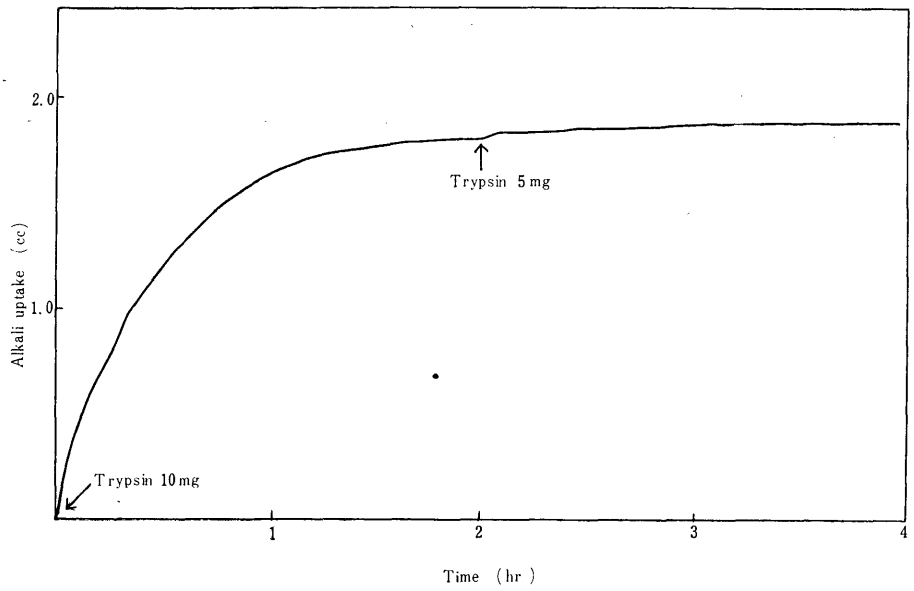

Fig. 2. Course of the tryptic digestion of the β-polypeptide chain of *Macaca mulatta* monkey hemoglobin

This alkali uptake curve resembles closely that of α-polypeptide chain,[14] however, in case of β-polypeptide chain, the reaction mixture was pretty cloudy even after hydrolysis for 4 hrs. Therefore, the turbidity was removed by centrifugation. When this transparent hydrolysate was adjusted to pH 6.4, what is called "core" contained in this hydrolysate precipitated. Then, "core" was removed by centrifugation.

In order to isolate and purify solube tryptic peptides from β-polypeptide chain of human hemoglobin, GUIDOTTI et al[6]. first fractionated them by countercurrent distribution method with the system of phenol, ethanol, HCl, and moreover, performed column chromatography with sephadex or Dowex 50 x 2. On the other hand, HILSE and BRAUNITZER[7] fractionated these soluble tryptic peptides by column chromatography with Dowex 1 x 2 as the adsorbent and various kinds of mixture of organic solvents as the developer and then refined them by paper chromatography. The author isolated and purified the soluble tryptic peptides

from β-polypeptide chain of *Macaca mulatta* monkey hemoglobin mainly according to RUDOLOFF and BRAUNITZER's method[18].

The elution curve is shown in Fig. 3.

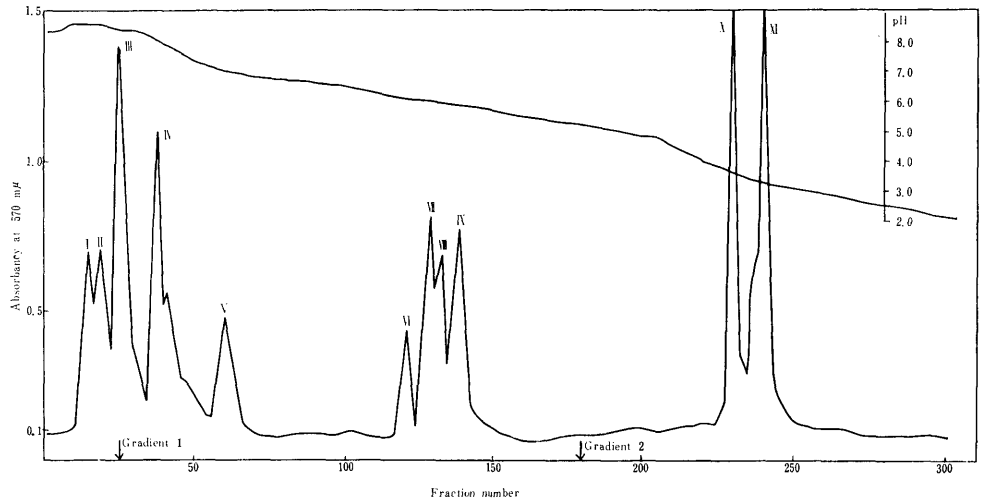

Fig. 3. Chromatography on Dowex 1 × 2 of the soluble tryptic peptides from the β-polypeptide chain of *Macaca mulatta* monkey hemoglobin

As shown in this figure, eleven peaks were recognized. The comparison of this result with HILSE and BRAUNITZER's[7] on β-polypeptide chain of human hemoglobin tells us that they resembles closely each other, though the buffer system used for development were different a little. However, the following fact is noticeable. In case of human hemoglobin the peak containing βT 13 was eluted near the neutral part, but in case of *Macaca mulatta* monkey hemoglobin the corresponding peak, Peak V in the Fig. 3, appeared in the alkali side. In order to inspect the purity of the peptide found in each peak, paper chromatography was carried out after the solution was concentrated to dryness under the reduced pressure.

Fig. 4 shows the peptides which were detected on this paper chromatogram by ninhydrin reaction and other specific amino acid reactions.

This was compared with HILSE and BRAUNITZER's report[7] on β-polypeptide chain of human hemoglobin. As in case of column chromatography, both have many common resemblances. However, Rf value of Spot VII-a which was supposed to correspond to βT 9 was extremely higher than that of human hemoglobin. The Rf value of Spot V-a which was supposed to correspond to βT 13 was also higher a little than that of human hemoglobin.

The peptides purified by paper chromatography was hydrolyzed with

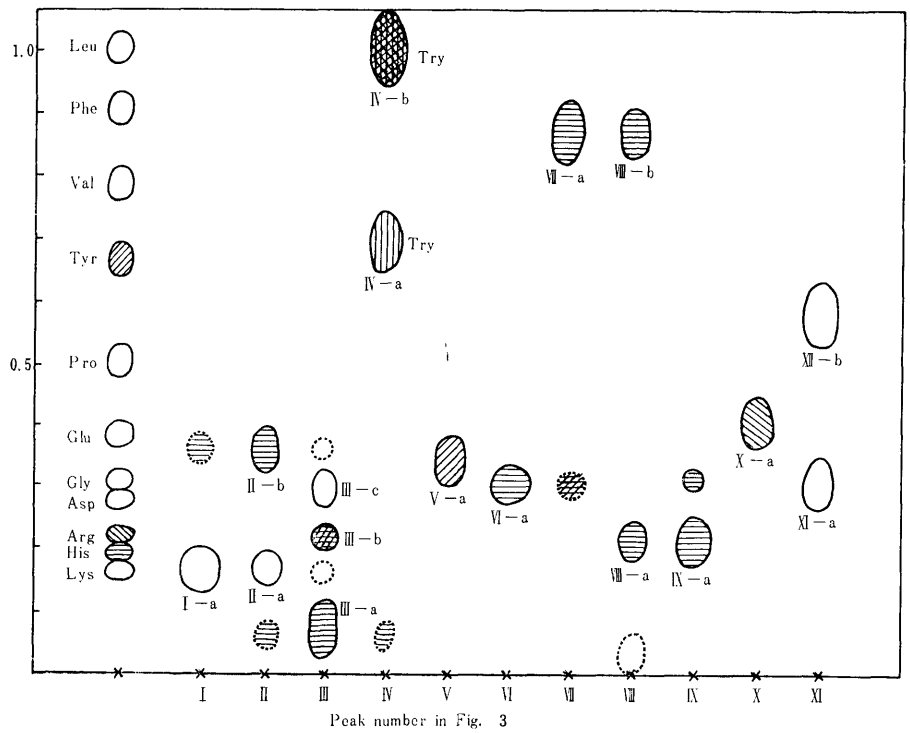

Fig. 4. Paper chromatogram of the soluble tryptic peptides from the β-polypeptide chain of *Macaca mulatta* monkey hemoglobin.

constant boiling point HCl at 105°C for 20 hrs or 40 hrs and their amino acids were analyzed. Table 1 shows the amino acid compositions of each peptide. The values in the Table were not corrected their losses resulting from hydrolysis.

Spot I-a (Rf Leu 0.18) was identical with lysine on the paper chromatogram and also the result of its amino acid analysis was lysine alone. Therefore, it is presumed to correspond to HßT 8 of tryptic digests of β-polypeptide chain from human hemoglobin. Spot II-a was same with Spot I-a. Peptide II-b (Rf Leu 0.38) resembled HβT 14 as to the elution position in column chromatography and on paper chromatogram. Its amino acid compositions were quite the same as those of HβT 14. Peptide III-a (Rf Leu 0.07) was identical with HβT 7. As to Spot III-b (Rf Leu 0.21), histidine and tyrosine reaction were seen on the paper chromatogram, however, their quantities were very small by amino acid analysis. It is presumed that this peptide may be βT 14 + 15 which remained by incomplete digestion of the peptide linkage between βT14 and βT 15. BRAUNITZER et al. recognized the same peptide. Peptide III-c (Rf Leu 0.30) was identical with HβT 6 both on chromatogram and in amino acid compositions. Peptide IV-a (Rf Leu 0.70) was the peptide containing tryptophan and resembled HβT 2 comparatively,

Table 1
Amino acid composition of the soluble tryptic peptides from the β-chain of *Macaca mulatta* monkey hemoglobin

	I – a	II – b	III – a	III – c	IV – a	IV – b	V – a	VI – a	VII – a	IX – a	X – a	XI – b
Lys	1.00	1.08	1.14	1.14	1.04		1.07	1.13	1.05	1.10		1.10
His		0.92	0.97						0.97	0.96		
Arg						1.11					1.02	
Met-O_2												+
Asp		1.02			1.02				3.89		1.99	2.94
Thr					1.86	0.99	0.89			0.91		
Ser									0.89			2.83
Glu						1.15	4.15			2.01	2.02	1.08
Pro						1.09	1.03			1.01		1.90
Gly		1.09	1.02		0.98				2.13		2.95	2.08
Ala		4.02	0.97		1.02		2.09		1.08		1.03	1.11
Val		2.83		0.85	0.95	1.16	1.04		0.90	0.93	2.95	0.97
Leu		1.02			1.02	2.74			4.08	1.05	0.99	1.05
Tyr						0.78	0.60	0.86				
Phe							0.91		0.96			3.00
Try					+	+						

but in amino acid compositions, the former had one mol more aspartic acid and one mol more threonine than the latter; on the contrary, the former had one mol less serine and one mol less alanine than the latter. This fact means that there are at least two differences in the amino acid sequences of the peptides between human and *Macaca mulatta* monkey hemoglobin. SHIKAYA[21] presumed the existence of the differences in this peptide of the two hemoglobins by fingerprinting method. Peptide IV-b (Rf Leu 0.99) was also the peptide containing tryptophan and besides, the reaction of arginine and tyrosine were found on the paper chromatogram. This peptide seemed to correspond to HβT 4, because of its Rf value on the paper chromatogram and its elution rate in column chromatography. However, this peptide had one mol more leucine and one mol less valine than HβT 4. Therefore, it is considered there is at least one difference in the amino acid sequence of this peptide between human and *Macaca mulatta* monkey hemoglobin. Though Peptide V-a (Rf Leu 0.34) was presumed to correspond to HβT 13 as mentioned previously, they were identical with each other neither on paper chromatogram nor in column chromatography. In its amino acid compositions it had one mol more glutamic acid and one mol less proline than HβT 13. About this difference, SHIKAYA[21] presumed in his report by fingerprinting method. Peptide VI-a (Rf Leu 0.30) was dipeptide consisted of histidine and tyrosine. It consisted with Tyr-His (HβT 15), C-terminal peptide in β-polypeptide chain of human

hemoglobin. Peptide VII-a (Rf Leu 0.85) was neutral peptide containing histidine. In its amino acid compositions, it had one mol more aspartic acid and one less alanine than HβT 9 and moreover its Rf value was pretty higher than that of HβT 9 on the paper chromatogram. Spot VIII-a was the same peptide with IX-a; Spot VIII-b, with VII-a. Peptide IX-a (Rf Leu 0.21) was histidine-positive peptide and had the same position on the chromatogram and the same amino acid compositions with HβT 1. The amino acid compositions of Peptide X-a (Rf Leu 0.40) was quite the same as those of HβT 3. From the result of amino acid analysis, it was known that Spot XI-a (Rf Leu 0.29) and Spot XI-b (Rf Leu 0.40) had the same amino acid compositions. As this peptide contained methionine, it is considered that according to the change of methionine, this peptide appears at the different position on the chromatogram. As to its amino acid compositions, it had one mol less threonine and one mol more serine than HβT 5. It makes us presume that there is at least one difference in amino acid sequence of this peptide between human hemoglobin and *Macaca mulatta* monkey hemoglobin. In Table 2, the amino acid compositions of soluble tryptic peptides of β-polypeptide

Table 2

Comparison between the amino acid compositions of the tryptic peptides from the β-chain of human and *Macaca mulatta* monkey hemoglobin

Peptide no	βT 1		βT 2		βT 3		βT 4		βT 5		βT 6		βT 7		βT 8		βT 9		βT 13		βT 14		βT 15	
	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H
Lys	1	1	1	1					1	1	1	1	1	1	1	1	1	1	1	1	1	1		
His	1	1											1	1			1	1			1	1	1	1
Arg					1	1	1	1																
Asp			①		2	2			3	3							④	3			1	1		
Thr	1	1	②	①			1	1		①									1	1				
Ser				①					③	②							1	1						
Glu	2	2			2	2	1	1	1	1									④	③				
Pro	1	1					1	1	2	2									①	②				
Gly			1	1	3	3			2	2			1	1			2	2			1	1		
Ala			①	②	1	1			1	1			1	1			①	②	2	2	4	4		
Val	1	1	1	1	3	3	①	②	1	1	1	1					1	1	1	1	3	3		
Met									1	1														
Leu	1	1	1	1	1	1	③	②	1	1							4	4	1	1	1	1		
Tyr							1	1											1	1			1	1
Phe									3	3							1	1						
Try			1	1			1	1																
Total	8	8	9	9	13	13	10	10	19	19	2	2	4	4	1	1	16	16	12	12	12	12	2	2

M means monkey hemoglobin. H means human hemoglobin. The values marked with ○ are those different between monkey and human hemoglobin.

chain in *Macaca mulatta* monkey hemoglobin are given and compared with those of human hemoglobin.

As shown in the Table, in the sequence of 108 amino acids of β-polypeptide chain of *Macaca mulatta* monkey hemoglobin, at least six parts are different from those of human hemoglobin. These exchanges of amino acids are possible to occur between serine and threonine, between alanine and aspartic acid, between threonine and serine, between valine and leucine, between alanine and aspartic acid and between proline and glutamic acid.

CONCLUSION

From *Macaca mulatta* monkey hemoglobin, β-polypeptide chain was isolated by countercurrent distribution method. Peptides soluble at pH 6.4 in the hydrolysate obtained by digestion of β-polypeptide chain with trypsin were isolated and purified by column chromatography with Dowex 1×2 and paper chromatography. The amino acid compositions of these peptides were examined. The comparison of the results obtained by the experiments described previously with those of human hemoglobin showed six differences between the two hemoglobins. This fact demonstrates that there are at least six differences in the sequence of 108 amino acids of β-polypeptide chain of *Macaca mulatta* monkey hemoglobin from human hemoglobin.

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REFERENCES

1) AGHER, R. and CROCKER, C. : *Biochim. Biophys. Acta* 9 : 704 (1952)
2) BRAUNITZER, G., GEHRING-MÜLLER, R., HILSCHMAN, N., HILSE, K., HOBOM, G., RUDLOFF, V., AND WITTMA-LIEBOLD, B. : *Hoppe-Seyler's Z Physiol. Chem.* 325 : 94 (1961)
3) CABANNES, R. AND SERAIN, C. : *C. R. Soc. Biol., Paris,* 151 : 943 (1957)
4) DRABKIN, D. L. : *J. Biol. Chem.* 164 : 703 (1946)
5) FINE, J. M., URIEL, J. AND FAURE, J : *Bull. Soc. Chim. Biol* 38 :649 (1956)
6) GUIDOTTI, G., HILL, J. R. AND KONIGSBERG, W. : *J. Biol. Chem.* 237 :2184 (1962)
7) HILSE, K. AND BRAUNITZER, G. : *Hoppe-Seyler's Z Physiol. Chem.* 329 : 8 (1962)
8) JEPSON, J. B. AND SMITH, I. : *Nature* 172 : 1100 (1953)
9) KONIGSBERG, W., GUIDOTTI, G., AND HILL, R. : *J. Biol. Chem.* 236 : PC 55 (1961)

10) MAITA, T. : *Acta Med. Nagasaki* 10 : 121 (1965)
11) MÄSIAR, P. : *Brartislav. Lèkarske Listy* 42 : 338 (1962)
12) MATSUDA, G., GEHRING-MÜLLER, R., AND BRAUNITZER,G. : *Biochem. Z.* 338 : 669 (1963)
13) MATSUDA, G. AND MAITA, T. : *J. Biochem.* 55 : 340 (1964)
14) MATSUDA, G., MAITA, T., TAKEI,H., MAEKAWA, T., SHIKAYA, T., MAEDA, K., AND FUJIWARA, M. : *Acta Med. Nagasaki* 8 : 28 (1963)
15) MATSUDA, G., OTA, H., AND YAMAGUCHI,M. : in preparation
16) REDFIELD, R. R. AND ANFINSEN, C. B. : *J. Biol. Chem.* 221 : 385 (1956)
17) RODNAN, G. P. AND EBAUGH, F. G. : *Proc. Soc. Exp. Biol., N. Y.*, 95 : 397 (1957)
18) RUDOLOFF, V. AND BRAUNITZER, G. : *Hoppe-Seyler's Z. Physiol. Chem.* 323 : 129 (1961)
19) SCHAPIRA, G. AND KRUH,J. : *Exp. Ann. Biochem. Med.* 13 : 285 (1951)
20) SCHROEDER, W. A., SHELTON, J. R., SHELTON, J. B., AND CORMICK,J. : *Proc. Natl. Acad. Sic.* 48 : 284 (1962)
21) SHIKAYA, T. : *Acta Med. Nagasaki* 10 : 77 (1965)
22) SMITH, D. B. : *Canad. J. Biochem.* 42 : 755 (1964)
23) SMITH, I. : *Nature* 171 : 43 (1953)
24) SMITH, I. : *Chromatographic Techniques* p. 60, William Heineman Medical Book Ltd., London (1958)
25) TEALE, F. W. J. : *Biochim. Biophys. Acta* 35 : 543 (1959)
26) YEMM, E. W. AND COCKING, E. C. : *Analyst* 80 : 209 (1955)
27) ZUCKERKANDL, E., JONES, R. T., AND PAULING, L. : *Proc. Natl. Acad. Sci.* 46 : 1349 (1960)
28) ZUCKERKANDL, E. AND SCHROEDER, W. A. : *Nature* 192 : 984 (1961)