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<th>The N-Terminal Structure of Adult Hemoglobin from Japanese Monkey (Macaca fuscata fuscata) and the N-Terminal Amino Acids of All the Tryptic Peptides from its α and β Polypeptide Chains</th>
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<td>Tanaka, Yoshiro</td>
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The N-Terminal Structure of Adult Hemoglobin from Japanese Monkey (Macaca fuscata fuscata) and the N-Terminal Amino Acids of All the Tryptic Peptides from its α and β Polypeptide Chains*

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The N-terminal structure of adult Japanese monkey (macaca fuscata fuscata) hemoglobin and the N-terminal amino acid analyses of the tryptic peptides from this hemoglobin were performed.

Hemoglobin, after removal of heme, was dinitrophenylated. The DNP-globin was hydrolyzed with HCl, and from the hydrolysate DNP-derivatives were extracted with ether or ethyl acetate. They were isolated and identified by silica gel-Celite column chromatography, and quantitative analyses were performed by spectrophotometry. Consequently, it was known that one mole of hemoglobin of the Japanese monkey consisted of two polypeptide chains whose N-terminal structure was Val-Leu (α chains) and two polypeptide chains whose N-terminal structure was Val-His-Leu (β chains). On the other hand, globin was separated into the subunits, α and β polypeptide chains by countercurrent distribution. Each polypeptide chain was hydrolyzed with trypsin. The hydrolyzed peptides were isolated and purified by column chromatography and paper chromatography. The N-terminal amino acids of the peptides thus isolated and purified were discussed by comparing with human hemoglobin. It was known that the N-terminal structure of βT2 peptide consisted of serine in the case of human hemoglobin, but asparagine in the case of Japanese monkey hemoglobin and that other 21 peptides has same N-terminal amino acids as those from human hemoglobin do, respectively.

INTRODUCTION

In this laboratory, hemoglobins from various animals have comparatively been studied. Evolutional problems of living organisms have generally been investigated with comparative studies of their physical shapes and habits, however, in the light of the fact that all the living organisms are of chemical constituents it is surely another good way...

*This work was presented at the 41st meeting of the Japanese Biochemical society, October 1968.
**田中義郎
for elucidation of the problems to compare those chemical constituents at molecular level among different species of living organisms. The recent information that the amino acid sequences of proteins are under the control of basic sequence of gene, DNA proves that it is one of the most available ways to investigate on the species’ specificity in the primary structure of proteins whose physiological functions are common. For this investigation hemoglobin is a convenient experimental material since it widely distributes among the animal kingdom and because it can be isolated and purified more easily than other proteins.

The structure of the heme part of hemoglobin, which was determined by H. Fisher so early as 1928, is known to be common to all the hemoglobins from various kinds of animals. With respect of the protein moiety, globin, hemoglobins from various kinds of animals are being studied since Braunitzer et al.43 and Konigsberg et al.93 worked out the primary structure of adult human hemoglobin.

Primate hemoglobins are generally studied with interest in how different they are from human hemoglobin. Zuckerkandl et al122, 23) presumed that chimpanzee hemoglobin was similar to human hemoglobin, and that the α and β polypeptide chains of gorilla hemoglobin were different from those of human hemoglobin in one amino acid residue, respectively. Buehner-Janusch et al.32 analyzed the amino acid compositions of the tryptic peptides from hemoglobins of such monkeys as the hylobates, the papio, the perodicticus, the galago, and the lumur, and then they compared the results with human hemoglobin. According to their report, considerably many differences are found among them. Concerning hemoglobins of macaca genus, the whole primary structure of rhesus monkey hemoglobin was determined in this laboratory.13) Rhesus monkey hemoglobin proved to be different from human hemoglobin at four positions in the α polypeptide chain and at eight positions in the β polypeptide chain. The Japanese monkey (macaca fuscata fuscata) which inhabits only on the Japanese Islands belongs to the same genus with the rhesus monkey. In an attempt to investigate on differences in the amino acid sequences of hemoglobins between these two species, the primary structure of Japanese monkey hemoglobin is studied in this laboratory.

The present author determined the N-terminal structure of adult Japanese monkey hemoglobin by the DNP method and furthermore determined the N-terminal amino acids of the tryptic peptides from the α and β polypeptide chains.

MATERIALS AND METHODS

1) Preparation of Globin

Hemoglobin was obtained by the method of Drabkin.52 The blood was added 3.8% citric acid solution as an anticoagulant and then was subjected to centrifugation at 3,000 r.p.m. for 10 minutes in order to
remove plasma. The erythrocytes obtained were washed three times with 0.9% NaCl solution and hemolyzed by the addition of an equal volume of deionized water and half a volume of toluene.

The hemolysate was stirred in the cold room overnight and centrifuged at 12,000 r.p.m. for 60 minutes. From the middle layer between the stroma and toluene, hemoglobin solution was obtained. For removal of heme from hemoglobin, 50ml of hemoglobin solution was dropped with stirring into HCl-acetone mixture (15ml of concentrated HCl, 500ml of acetone) which was cooled at $-20^\circ$C. Globin precipitated in the acetone solution was isolated by centrifugation and then washed three times with a mixture of HCl and acetone. The globin thus obtained was dissolved in deionized water. It was dialyzed against deionized water and finally lyophilized.

2) Separation of $\alpha$ and $\beta$ Polypeptide Chains of the Globin by Countercurrent Distribution

The globin was separated into $\alpha$ and $\beta$ polypeptide chains with a mixture of 1650 ml of sec-butanol containing 1.56 g of trichloroacetic acid, 225 ml of propionic acid, and 1305 ml of water on an automatic countercurrent distribution machine with 100 tubes of 20 ml capacity, Shibata CDA-100; 500 mg of the globin was dissolved in 80 ml of the above described solvent, separated into the upper and lower phases, and then placed in the four tubes of the machine, Tube No. 4 to No. 7. 150 upper phase transfers were carried out by setting the machine for a 30-second shifting time and a 20-minute settling time. The solvent in the tubes was made transparent by the addition of 0.5 ml of 50% ethanol, and then the absorbance at 280 m\( \mu \) of each tube was measured. The solvents at the peaks were combined and dialyzed against deionized water before lyophilization.

3) Preparation of the Tryptic Peptides from the Polypeptide Chains

In 50 ml of 8 M urea solution, 1g of the $\alpha$ or $\beta$ polypeptide chain was dissolved and denatured at 60°C for 45 minutes. After removal of urea by dialysis, the polypeptide chain was suspended in 80 ml of deionized water and brought to pH 8.0 by the addition of 0.1 N NaOH. To this solution was added 15 mg of trypsin (Worthington Biochemical Corp. Twice crystallized) which was dissolved in 1/16N HCl and removed its chymotryptic activity. Hydrolysis was carried out at $37^\circ$C for 4 hours, the pH being maintained at 8.0. The hydrolysate was adjusted to pH 6.4 with 1 N acetic acid and then the insoluble peptide was precipitated. The soluble peptides and insoluble peptides were separated from each other by centrifugation. Isolation and purification of the soluble peptides were performed as follows by column and paper chromatographies.

a) Column Chromatography of the Soluble Tryptic Peptides on Dowex
The resin (Dowex 1×2, 200–400 mesh) was washed with 1 N NH₄OH, water, acetic acid, and water successively, and finally sus-
pended in the starting buffer. It was loaded in a column (2.0×150 cm). The column was further equilibrated with the starting buffer. The sample, the soluble peptide after adjusted to pH 10.0 with NaOH, was placed on the column. As a developer was used the acetate buffer containing organic bases such as pyridine, collidine, picoline, and lutidine. In addition, gradient elution with acetic acid was employed. Elution was carried out at a flow rate of 180–200 ml per hour, the column being kept at 37°C. The eluate was collected in 18 ml-fractions. According to the method of YEMM and COCKING, a 0.2 ml portion from each fraction subsequent to alkali hydrolysis was subjected to the ninhydrin color reaction. The eluate at each peak in the ninhydrin color reaction was dried under reduced pressure below 30°C and disso-
lved in 7 ml of deionized water.

b) Paper Chromatography of the Soluble Tryptic Peptides

Toyo filter paper No. 50 was used. Development was performed by descending method. The developer used was the upper phase of a mixture of n-butanol, acetic acid, and water (4:1:5). Peptides were detected by spraying 0.2% ninhydrin-n-butanol solution and by heating to color with an iron. Besides, PAULY’S reaction, SAkAGUCHI’S reac-
tion, EHRlich’S reaction, α-nitrosonaphtol reaction were carried out on the paper chromatogram for identification of peptides. The peptides were eluted with 5% acetic acid and the eluates were dried under reduced pressure.

4) Analysis of the N-terminal structure of the globin by the DNPmethod

a) Dinitrophenylation of the globin

In 75ml of deionized water was suspended 500mg of the globin and to this was added 0.1N NaOH to be pH 9.0. This suspension was further added 100 mg of Na₂CO₃ and 0.5 ml of dinitrofluorobenzene (DNFB) and stirred for 3 hours at 40°C. At the termination of the reaction, the solution was brought to pH 2.0 by the addition of 1N HCl. The DNP-
globin, which was recognized as a yellow precipitate, was isolated by centrifugation and washed 3 times with 0.1 N HCl. It was thorou-
gly washed on the glass filter with acetone and ether, and then dried by allowing to stand overnight at 50°C in an incubator.

b) Hydrolysis of the DNP-globin and extraction of DNP-derivatives from the hydrolysate

In 15ml of constant boiling point HCl (twice distilled) was sus-
pended 100mg of the dried DNP-globin. This suspension was hydrolyzed under refluxing for an appropriate time. DNP-derivatives were extra-
ected from the hydrolysate with ether and ethyl acetate by the procedure illustrated in Fig. 1. The ether or ethyl acetate extracts were dried under reduced pressure.

Fig. 1. Procedure of extraction of the DNP-derivatives from the hydrolysate of DNP-globin.

c) Silica gel-Celite column chromatography for the DNP-derivatives

Column Chromatography for the DNP-derivatives was performed by the modification of the method of Green et al., in which silica gel-Celite was used as an adsorbent. Silica gel (100 mesh, Mallinckrodt Chemical Work, U.S.A.) and Celite 545 (Wako Chemical Industry, Ltd.) was mixed at the rate of 2 to 1, and the mixture was loaded in a column (0.9×20 cm) at a height of 15cm. The column was washed with 5 ml of ether, 10 ml of ether-acetone mixture (1:1), 2ml of ether, and 7ml of ligroin, successively and was finally equilibrated with 7ml of the starting developer.

The DNP-derivative dissolved in 3 ml of the appropriate solvent was applied to the aforeprepared column. The developer used was the mixture containing ligroin, acetic acid, acetone, formic acid, and ethyl acetate. Movement of the yellow band of the DNP-derivatives in the column was recorded every 7ml of the developer. The isolated yellow band was taken out and eluated on a glass filter with ether-alcohol mixture (4:1). The eluate was dried under reduced pressure.

d) Estimation of the DNP-derivatives
The DNP-derivatives isolated by column chromatography were dissolved in an aliquot of glacial acetic acid and their absorbance at 340 m\(\mu\) were measured. Regarding the molecular extinction coefficient of the DNP-derivatives at 340 m\(\mu\) as 16,100, their quantities were calculated according to the following formula.

\[
\text{DNP-derivative (mole) = A} \times 6.2 \times \frac{a}{100}
\]

In the formula, \(a\) denotes the quantity (ml) of the acetic acid in which a DNP-derivative was dissolved, and \(A\), its absorbance at 340 m\(\mu\).

e) Identification of the DNP-derivatives

Each DNP-derivative after dissolved in 4ml of constant boiling point HCl was hydrolyzed at 105°C for 24 hours in a sealed tube. The hydrolysate was extracted with three 10 ml-portions of ether, and then the ether extract after dried under reduced pressure was subjected to the paper chromatography in order to identify and estimate its DNP-amino acids. The aqueous phase was also subjected to an amino acid analyzer, Hitachi KLA-2 in order to identify and estimate its amino acids.

5) N-terminal amino acid analysis of the tryptic peptides by the DNP method

In 10 ml of 1% NaHCO\(_3\) solution was dissolved 0.2–0.5 \(\mu\)mole of tryptic peptides, and to this was added 0.05 ml of dinitrofluorobenzen (DNFB). The mixture was stirred at 40°C for 2 hours. The excess of DNFB was removed by extraction with three 10 ml-portions of ether. The aqueous phase was acidified with HCl and extracted with three 10 ml-portions of ether. By this treatment, dinitrophenol (DNP) which was a byproduct in dinitrophenylation was extracted in the ether phase. The DNP-tryptic peptides in the aqueous phase, after dried under reduced pressure, were dissolved in constant boiling HCl and hydrolyzed at 105°C for 20 hours in a sealed tube. From this hydrolysate, DNP-amino acids were extracted with three 10 ml-portions of ether, and dried under reduced pressure in a heart-shaped flask, a modification of MILLS' apparatus. The flask was connected to a cold finger and completely aspirated by a vacuum pump in order to remove as much dinitrophenol (DNP) as possible. Identification of DNP-amino acids was performed as follows by two dimensional paper chromatography. The sample dissolved in a small portion of acetone was applied on a sheet of Toyo filter paper No. 50 (40×40 cm). Development was performed by the ascending method with the upper phase of n-butanol and 1N NH\(_4\)OH (1 : 1) for the first dimension and with 1.5 M phosphate buffer for the second dimension. The DNP-amino acids were estimated by measuring the absorbance at 360 m\(\mu\) of the eluates which were eluted with 5 ml of warm water from the yellow spots on the paper chromatogram.

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RESULTS AND DISCUSSION

1) The N-terminal structure of the hemoglobin from the Japanese monkey

Globin was prepared by removal of heme from hemoglobin of Japanese monkeys (*macaca fuscata fuscata*). It was dinitrophenylated in aforementioned method; 510 mg of dry DNP-globin was obtained from 500 mg of globin. Three 100 mg-portions of the DNP-globin were hydrolyzed with constant boiling point HCl under refluxing for 1 hour, 6 hours, and 20 hours, respectively. From the hydrolysates, DNP-derivatives were extracted by ether or ethylacetate and then they were isolated by silica gel-Celite column chromatography. The ether extracts were dissolved in 3 ml of Solvent I, a mixture of 0.06 ml of acetic acid, 0.3 ml of acetone and 2.6 ml of ligroin, and applied on the column. Development carried out by using 8AA4AL. The movement of the yellow band was given in Fig. 2-a. As seen in this figure, two yellow bands, A and B, were separated at the time when 21 ml of the ether extract was applied.

![Silica gel-Celite chromatography of ether-extracted DNP-derivatives from the hydrolysate of the DNP-globin.](image)

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**Fig. 2.** Silica gel-Celite chromatography of ether-extracted DNP-derivatives from the hydrolysate of the DNP-globin.

8AA4AL: The mixture of acetic acid (8 ml) and acetone (4 ml) was added ligroin so that the whole volume was 100 ml.

2F8EL: The mixture of formic acid (2 ml) and ethyl acetate (8 ml) was added ligroin so that the whole volume was 100 ml.
The mobility of B-band was similar to that of DNP-amino acids (DNP-Val, DNP-Leu, DNP-Ileu) of IV group which were isolated by the chromatography using the system of Green et al. The yellow substance taken out from the column was eluted with ether-alcohol mixture and subjected to the two dimensional paper chromatography. As a result, only a spot which was identical with the standard DNP-Val was recognized. Therefore, this substance was identified as DNP-Val. In the similar way, A-band was subjected to the two dimensional paper chromatography. Two spots were found. One accorded with dinitroaniline (DNA) and the other accorded with none of the DNP-amino acids. Therefore, DNP-derivatives contained in A-band were dissolved in 3 ml of Solvent I and subjected to column chromatography with 2F8EL developer. The result was given in Fig. 2-b. It was separated into two yellow bands, C and D. D-band corresponded to DNA both in the mobility in the chromatography with this same system and in the location in the two dimensional paper chromatography. C-band corresponded to none of the DNP-amino acids on the paper chromatogram. Therefore, an aliquot of C-band was hydrolyzed with constant boiling HCl at 105°C for 24 hours in a sealed tube. Yellow substance was extracted with ether. The ether phase was subjected to paper chromatography, and the aqueous phase after dried was subjected to an amino acid analyzer. Consequently,

Fig. 3. Silica gel-Celite chromatography of ethylacetate-extracted DNP-derivatives from the hydrolysate of the DNP-globin
8AA4AL; See Fig. 2.
3AA15AL; The mixture of acetic acid (3 ml) and acetone (15 ml) was added ligroin so that the whole volume was 100 ml.
4AA20AL; The mixture of acetic acid (4 ml) and acetone (20 ml) was added ligroin so that the whole volume was 100 ml.
DNP-Val was only recognized in the ether phase. Its quantity was 0.130 μmole. In the aqueous phase, leucine was only recognized. Its quantity was 0.163 μmole. These facts suggest that C-band could be identified as DNP-Val-Leu.

The ethylacetate extract was dissolved in 3ml of Solvent II, a mixture of 0.6ml of acetic acid and 2.4ml of benzene, and then isolated by column chromatography with 8AA4AL, the starting developer. As seen in Fig. 3, the yellow band did not move in the development with 21ml of 8AA4AL. Therefore, development with 14 ml of 3AA15AL and 14 ml of 4AA20AL followed it. As a result of it, three yellow bands were separated. E-band gave many spots on the two-dimensional paper chromatography. However, from the ether extract from the HCl hydrolysate of E-band, nothing like the yellow substance was extracted. On the contrary, from the aqueous phase, ε-mono-DNP-Lys and many free amino acids were found, suggesting that E-band was a mixture of ε-mono-DNP-Lys and peptides containing ε-mono-DNP-Lys and that it had nothing to do with the N-terminal structure of the globin. As for F-band, only one spot was recognized on the paper chromatogram. The ether extract of F-band subsequent to HCl hydrolysis contained only DNP-Val, and the aqueous phase, only imid-mono-DNP-His which was eluted with about 50 ml of buffer, pH 5.28 on a 15 cm column of the automatic amino acid analyzer. Consequently, the DNP-derivative of F-band was identified as a dipeptide of DNP-Val-His. G-band also gave one spot on the paper chromatogram. After HCl hydrolysis of it, DNP-Val, imid-mono-DNP-His, and Leu were recognized in the same mole number. Therefore, in due consideration of the results on F-band, the DNP-derivative of G-band was identified as DNP-Val-His-Leu.

DNP-Val, DNP-Val-Leu, DNP-Val-His, and DNP-Val-His-Leu thus obtained were analyzed quantitatively by spectrophotometry. Their values were calculated per 1 mole of the DNP-globin, regarding 100 mg

<table>
<thead>
<tr>
<th>Hydrolysis time (hr)</th>
<th>DNP-Val</th>
<th>DNP-Val-Leu</th>
<th>DNP-Val-His</th>
<th>DNP-Val-His-Leu</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.481</td>
<td>1.382</td>
<td>0.842</td>
<td>0.312</td>
<td>3.017</td>
</tr>
<tr>
<td>6</td>
<td>2.121</td>
<td>0.533</td>
<td>0.364</td>
<td>0.108</td>
<td>3.128</td>
</tr>
<tr>
<td>20</td>
<td>3.647</td>
<td>0.229</td>
<td>0.108</td>
<td>–</td>
<td>3.984</td>
</tr>
</tbody>
</table>

These values are expressed as molar ratios
DNP-globin, 100mg, is assumed to be 1.14 μmole.
of the DNP-globin as 1.14 μmole.\textsuperscript{16} Table I gives the results. The percentage recoveries of the DNP-derivatives were not corrected except that that of DNP-Val obtained by 20 hour hydrolysis was corrected to be 90%. As seen in the table, Japanese monkey hemoglobin turned out, just as in the case of human hemoglobin, to be composed of two polypeptide chains whose N-terminal structure was Val-Leu, and two polypeptide chains whose N-terminal structure was Val-His-Leu.

2) The N-terminal structure of the $\alpha$ and $\beta$ polypeptide chains

The globin was separated into its subunits, $\alpha$ and $\beta$ polypeptide chains by countercurrent distribution. Fig. 4 shows the result.

![Fig. 4 Countercurrent distribution of the globin from Japanese monkey hemoglobin](image)

Analyses of the N-terminal structures of the polypeptide chains were performed in quite the same ways as in the case of the globin. The analysis values were converted into those per 3,300 g (2 moles) of a polypeptide chain and given in Table II. As seen in this table, the $\alpha$ polypeptide chains have the N-terminal structure of Val-Leu and the $\beta$ polypeptide chains, that of Val-His-Leu. The values of DNP-Val, DNP-Val-His, and DNP-Val-His-Leu obtained after one hour hydrolysis suggest that the purity of each chain separated by countercurrent distribution of this system was over 93%.

3) The N-terminal amino acids of the tryptic peptides from $\alpha$ and $\beta$ polypeptide chains

The $\alpha$ and $\beta$ polypeptide chains were digested with trypsin subse-
Table II

Number of N-terminal residues in the α and β polypeptide chains of Japanese monkey hemoglobin

<table>
<thead>
<tr>
<th></th>
<th>Hydrolysis time (hr)</th>
<th>DNP-Val</th>
<th>DNP-Val-Leu</th>
<th>DNP-Val-His</th>
<th>DNP-Val-His-Leu</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>α chain</td>
<td>1</td>
<td>0.222</td>
<td>1.230</td>
<td>0.091</td>
<td>0.030</td>
<td>1.573</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.720</td>
<td>0.091</td>
<td>—</td>
<td>—</td>
<td>1.811</td>
</tr>
<tr>
<td>β chain</td>
<td>1</td>
<td>0.203</td>
<td>0.103</td>
<td>0.941</td>
<td>0.213</td>
<td>1.560</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.694</td>
<td>—</td>
<td>0.061</td>
<td>—</td>
<td>1.755</td>
</tr>
</tbody>
</table>

These values are expressed as molar ratios.

The molecular weight of the polypeptide chain is assumed to be 33,000.

sequently to denaturation in 8 M urea. Peptides in the digest were first fractionated by the precipitating method at pH 6.4 into the soluble tryptic peptides and the insoluble one called "core". The soluble tryptic peptides were isolated and purified both by column chromatography of Dowex 1×2 with acetate buffer containing such organic bases as pyridine, collidine, and picoline and by paper chromatography developed with the mixture of n-butanol, acetic acid and water. The results are shown in Figs. 5, 6, 7, and 8.

A portion of each peptide thus isolated was hydrolyzed with HCl and then subjected to amino acid analysis. As described above, iden-

Fig. 5. Column chromatogram of the soluble tryptic peptides from the α chain of Japanese monkey hemoglobin. Column: 2.0×150 cm, Starting developer: 1% pyridine, 1% collidine acetate buffer, pH 9.0. Step 2: 1% pyridine 1% collidine acetate buffer, pH 8.5. Gradient 1: Mixing chamber, 1500 ml of 1% pyridine 1% lutidine 1% picoline acetate buffer, pH 7.5. Upper chamber, 0.08 N acetic acid. Gradient 2: Upper chamber 1.0 N acetic acid.
Fig. 6. "Paper" chromatogram of the soluble tryptic peptides from the α chain of Japanese monkey hemoglobin.

Fig. 7. Column chromatogram of the soluble tryptic peptides from the β chain of Japanese monkey hemoglobin. Column: 2.0 x 150 cm. Starting developer: 1% pyridine 1% collidine acetate buffer, pH 9.0. Step 2: 1% pyridine 1% collidine acetate buffer, pH 8.5. Gradient 1: Mixing chamber, 1500 ml of 1% pyridine 1% lutidine 1% picoline acetate buffer, pH 7.5 Upper chamber, 0.08 N acetic acid. Gradient 2: Upper chamber, 1.0 N acetic acid. 
Fig. 8. Paper chromatogram of the soluble tryptic peptides from the \( \beta \) chain of Japanese monkey hemoglobin.

tification, estimation of the purity, and estimation of quantity were performed. Concerning amino acid compositions of these peptides, Terag\(^{20}\) and Tachikawa\(^{19}\) have already reported. The peptides on the paper chromatogram in Figs. 6 and 8 are numbered according to their reports, in which they were designated in the light of those of human hemoglobin.

The insoluble tryptic peptide was thoroughly washed with sodium acetate buffer, pH 6.4, suspended in deionized water, and lyophilized. A portion of the insoluble tryptic peptide thus prepared was hydrolyzed with HCl and submitted to amino acid analysis, and then the N-terminal structure was analyzed.

Dinitrophenylation of the insoluble tryptic peptides was carried out in NaHCO\(_3\) solution and then the excess DNFB was removed by ether extraction. The aqueous phase acidified with HCl was again subjected to ether extraction. By this treatment, all the DNP-tryptic peptides containing lysine and arginine remained in the aqueous phase, and dinitrophenol (DNP), the byproduct during the reaction, could be removed in the ether phase. This treatment was convenient because dinitrophenol was obstructive to paper chromatography for identification of DNP-amino acids. DNP-peptides were hydrolyzed with HCl and then DNP-amino acids due to their N-terminus were extracted with ether. They were isolated and identified by the two dimensional paper chromatography and estimated by spectrophotometry. Fig. 9 shows the
N-terminal amino acids and amino acid compositions of all the tryptic peptides from the α-polypeptide chain of Japanese Money hemoglobin

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>DNP-amino acids found /mole</th>
<th>Amino acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>αT1</td>
<td>DNP-Val 0.72</td>
<td>Val-(Asp 1, Ser 1, Pro 1, Ala 1, Leu 1)-Lys</td>
</tr>
<tr>
<td>αT2</td>
<td>DNP-Ser 0.71</td>
<td>Ser-(Asp 1, Val 1)-Lys</td>
</tr>
<tr>
<td>αT3</td>
<td>DNP-Ala 0.56</td>
<td>Ala-(Gly 1, Ala 1, Try 1)-Lys</td>
</tr>
<tr>
<td>αT4</td>
<td>DNP-Val 0.63</td>
<td>Val-(His 1, Glu 3, Gly 4, Ala 3, Leu 1, Tyr 1)-Arg</td>
</tr>
<tr>
<td>αT5</td>
<td>DNP-Met 0.34, DNP-MetO 0.12</td>
<td>Met-(Thr 2, Ser 1, Pro 1, Leu 1, Phe 2)-Lys</td>
</tr>
<tr>
<td>αT6</td>
<td>DNP-Thr 0.68</td>
<td>Thr-(His 2, Asp 1, Ser 2, Glu 1, Pro 1, Gly 1, Ala 1, Val 1, Leu 1, Tyr 1, Phe 2)-Lys</td>
</tr>
<tr>
<td>αT7</td>
<td>DNP-Gly 0.43</td>
<td>Gly-(His 1, Gly 1)-Lys</td>
</tr>
<tr>
<td>αT8</td>
<td></td>
<td>Lys</td>
</tr>
<tr>
<td>αT9</td>
<td>DNP-Val 0.71</td>
<td>Val-(His 3, Asp 5, Thr 1, Ser 2, Pro 1, Gly 1, Ala 6, Val 2, Met 1, Leu 5)-Lys</td>
</tr>
<tr>
<td>αT10</td>
<td>DNP-Leu 0.83</td>
<td>Leu-Arg</td>
</tr>
<tr>
<td>αT11</td>
<td>DNP-Val 0.65</td>
<td>Val-(Asp 2, Pro 1, Val 1, Phe 1)-Lys</td>
</tr>
<tr>
<td>αT12,13</td>
<td>DNP-Leu 0.71</td>
<td>Leu-(Lys 1, His 3, Asp 1, Thr 4, Ser 5, Glu 1, Pro 2, Ala 6, Cys 1, Val 4, Leu 9, Phe 2)-Lys</td>
</tr>
<tr>
<td>αT14</td>
<td>di DNP-Tyr 0.34</td>
<td>Tyr-Arg</td>
</tr>
</tbody>
</table>

Fig. 9 Paper chromatogram of the DNP-amino acids and DNP-peptides
N-terminal amino acids and amino acid compositions of all the tryptic peptides from the \( \beta \)-polypeptide chain of Japanese monkey hemoglobin

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>DNP-amino acids found</th>
<th>Amino acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \beta T1 )</td>
<td>DNP-Val 0.72</td>
<td>Val-(His 1, Thr 1, Glu 2, Pro 1, Leu 1)-Lys</td>
</tr>
<tr>
<td>( \beta T2 )</td>
<td>DNP-Asp 0.63</td>
<td>Asp-(Ser 2, Gly 1, Ala 1, Val 1, Leu 1, Trp 1)-Lys</td>
</tr>
<tr>
<td>( \beta T3 )</td>
<td>DNP-Val 0.68</td>
<td>Val-(Asp 2, Glu 2, Gly 3, Ala 1, Val 2, Leu 1)-Arg</td>
</tr>
<tr>
<td>( \beta T4 )</td>
<td>DNP-Leu 0.80</td>
<td>Leu-(Thr 1, Glu 1, Pro 1, Val 2, Leu 1, Tyr 1, Trp 1)-Arg</td>
</tr>
<tr>
<td>( \beta T5 )</td>
<td>DNP-Phe 0.66</td>
<td>Phe-(Asp 3, Ser 3, Glu 1, Pro 2, Gly 2, Ala 1, Val 1, Met 1, Leu 1, Phe 2)-Lys</td>
</tr>
<tr>
<td>( \beta T6 )</td>
<td>DNP-Val 0.80</td>
<td>Val-Lys</td>
</tr>
<tr>
<td>( \beta T7 )</td>
<td>DNP-Ala 0.63</td>
<td>Ala-(His 1, Gly 1)-Lys</td>
</tr>
<tr>
<td>( \beta T8 )</td>
<td>DNP-Val 0.64</td>
<td>Val-(His 1, Asp 4, Ser 1, Gly 2, Ala 1, Leu 4, Phe 1)-Lys</td>
</tr>
<tr>
<td>( \beta T9 )</td>
<td>DNP-Gly 0.39</td>
<td>Gly-(Lys 1, His 2, Asp 3, Thr 1, Ser 1, Glu 3, Pro 1, Ala 1, Cys 1, Val 1, Leu 3, Phe 2)-Lys</td>
</tr>
<tr>
<td>( \beta T10,11 )</td>
<td>DNP-Leu 0.51</td>
<td>Leu-(His 2, Asp 1, Gly 2, Ala 1, Cys 1, Val 2, Leu 3, Phe 1)-Lys</td>
</tr>
<tr>
<td>( \beta T12 )</td>
<td>DNP-Glu 0.58</td>
<td>Glu-(Thr 1, Glu 3, Pro 1, Ala 2, Val 1, Tyr 1, Phe 1)-Lys</td>
</tr>
<tr>
<td>( \beta T13 )</td>
<td>DNP-Val 0.55</td>
<td>Val-(His 1, Asp 1, Gly 1, Ala 4, Val 2, Leu 1)-Lys</td>
</tr>
<tr>
<td>( \beta T14 )</td>
<td>di DNP-Tyr 0.33</td>
<td>Tyr-His</td>
</tr>
</tbody>
</table>

From \( \alpha T12,13 \) and \( \beta T10,11,12 \), the so-called insoluble tryptic peptides, many DNP-amino acids were detected. In the case of \( \alpha T12,13 \), however, the N-terminal residue is considered to be leucine since DNP-Leu was overwhelmingly more than others. \( \beta T10,11,12 \) contained plenty of DNP-Leu and DNP-Gly. Since it is said that DNP-Gly shows weak resistance against HCl hydrolysis and that its recovery is smaller than others\(^{14} \), this result indicates that \( \beta T10,11,12 \) contains the peptide whose N-terminus is glycine as much as or more than the peptide whose N-terminus is leucine. It turns out that \( \beta T10,11,12 \) consists of two kinds of peptides whose N-termina are composed of glycine and leucine, respectively. Tachikawa, in his study of the \( \beta \) polypeptide chain by amino-ethylolation and tryptic hydrolyses, found three peptides, \( \beta T10,11 \), \( \beta T12a \) and \( \beta T12b \) instead of an insoluble tryptic peptide\(^{19} \). It is considered that the peptide whose N-terminal amino acid was glycine is identical.
with $\beta$T10,11 and that the peptide whose N-terminal amino acid was leucine is identical with $\beta$T12.

The N-terminal amino acids of the tryptic peptides thus investigated were compared with those from human hemoglobin. They are quite the same with each other except $\beta$T2. The N-terminal amino acid of $\beta$T2 from Japanese monkey hemoglobin was identified as DNP-Asp, which originated from asparagine, since $\beta$T2 peptide was basic from the result of paper electrophoresis. On the other hand, the N-terminal amino acid residue of $\beta$T2 of human hemoglobin is serine.

**CONCLUSION**

The N-terminal structure of Japanese monkey (*macaca fuscata fuscata*) was analyzed by the DNP method. It was known that Japanese monkey hemoglobin was composed of four polypeptide chains i.e. two $\alpha$ polypeptide chains ($\alpha$ chains) whose N-terminal structure was Val-Leu and two $\beta$ polypeptide chains ($\beta$ chains) whose N-terminal structure was Val-His-Leu. Next, these polypeptide chains were separated by countercurrent distribution and tryptic peptides were obtained. The N-terminal structures of all the tryptic peptides were determined. The results were compared with those from human hemoglobin. One difference was found in $\beta$T2 peptide; The N-terminal amino acid of $\beta$T2 is serine in the case of human hemoglobin but asparagine in the case of Japanese monkey hemoglobin.

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