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<td>Tachikawa, Isamu</td>
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Isolation of All Tryptic Peptides from the Aminoethylated \( \beta \) Polypeptide Chain of Adult Hemoglobin from Japanese Monkey (Macaca fuscata fuscata) and their Amino Acid Compositions*

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Hemoglobin prepared from red blood cells of adult Japanese Monkeys (Macaca fuscata fuscata) was removed heme so that globin was obtained. The globin was separated into \( \alpha \) and \( \beta \) polypeptide chains by countercurrent distribution with the system of sec-butanol containing trichroloacetic acid, propionic acid, and water. The \( \beta \) polypeptide chain was aminoethylated and hydrolyzed with trypsin. The tryptic peptides were isolated and purified by column chromatography, paper chromatography, and high voltage paper electrophorsis. Analyses of amino acid compositions of these peptides were performed after HCl hydrolysis.

In comparison with human hemoglobin A, Japanese monkey hemoglobin turned out to be different at at least seven positions of the five peptides from the \( \beta \) polypeptide chain. Similarly, in comparison with rhesus monkey hemoglobin, only one difference in the amino acid composition could be found between rhesus monkey (Macaca mulatta) and Japanese monkey which belong to the common genus.

INTRODUCTION

Anfinsen22, in his book, "Molecular Basis of Evolution", proposed quite a new standpoint against the traditional theory of evolution to elucidate evolutilional problems by comparative studies of shapes and habits of living organisms. He, by comparing the structures of proteins from various living organisms, tried to presume the evolutilional process of genes which control protein biosynthesis. It proves to be one of the admirable keys for elucidation of evolutilional problems at the present stage it has already been known not only that amino acid sequences of proteins are controlled by gene, DNA, but also that the primary structures of various proteins are able to be determined.

From the above notion, hemoglobin is one of the most suitable

\*This work was presented at the 41st meeting of The Japanese Biochemical Society, October 1968.

**立川 勇
research materials. Since the primary structure of human hemoglobin A was worked out by BRAUNITZER et al. and KONIGSBERG et al., hemoglobins from various kinds of animals have been studied besides those from horse, human fetal, and bovine fetal.

Concerning primate hemoglobins, it was presumed that the primary structure of chimpanzee hemoglobin would be quite similar to that of human hemoglobin. ZUCKERKANDL et al. who analyzed amino acid compositions of the tryptic peptides of gorilla hemoglobin, reported that it would be different from those of human hemoglobin in one residue of both α and β polypeptide chains, respectively. In addition, BUETTNER-JANUSCH et al. studied on the partial amino acid compositions of the tryptic peptides of hemoglobins from the hylobates, the papio, the perodicticus, the galogo, and the lumur, and then compared the results with the corresponding peptides of human hemoglobin. According to their reports, pretty many differences could be found among these primate hemoglobins.

In this laboratory, the whole primary structure of rhesus monkey (macaca mulatta) hemoglobin was already determined. According to this result, 12 differences in the amino acid sequence were confirmed between human and rhesus monkey hemoglobins, that is, four in the α polypeptide chain and eight in the β polypeptide chain.

Japanese monkeys (macaca fuscata fuscata) live only in the Japanese Islands and belong to the same genus with the previously-studied rhesus monkey. It is therefore of great interest to investigate how different the primary structure of these two species are. The present paper describes the determination of the amino acid compositions of all the tryptic peptides from the β polypeptide chain of Japanese monkey hemoglobin in addition to the methods of isolation and purification of tryptic peptides obtained by digestion of the aminoethylated β polypeptide chain.

MATERIALS AND METHODS

1) Preparation of hemoglobin

According to the method of DRABKIN, hemoglobin was obtained from blood drawn from adult Japanese monkeys (macaca fuscata fuscata). Blood added 3.8% sodium citrate as the anticoagulant was subjected to centrifugation (3,000 r.p.m. 10 minutes) to remove plasma. Red blood cells were washed three times with 0.9% NaCl. To this was added an equal volume of deionized water and half a volume of toluene. This solution was stirred overnight in the cold for hemolysis. The hemolysate was centrifuged for 1 hour at 12,000 r.p.m. and then the hemoglobin solution was obtained from the middle layer between the toluene and the membrane components of blood cells.
2) Preparation of globin

By the method of ANSON and MIRSKEY, hemoglobin was converted to globin. First, 50 ml of hemoglobin solution (10g/dl) was added with stirring to a HCl-acetone mixture (15 ml of conc. HCl + 500 ml of acetone) and then the mixture was stirred for 15 minutes more. By this treatment, hemoglobin was gradually removed its heme and globin precipitated. The globin was subjected to centrifugation, washed three times with the HCl-acetone mixture, and then dissolved in the appropriate quantity of deionized water. It was dialyzed against deionized water and finally lyophilized.

3) Countercurrent distribution for separation of α and β polypeptide chains

The machine used for countercurrent distribution was Shibata CDA Model 100 with 100 tubes of 20 ml capacity. All the procedures were carried out at 25°C in the constant temperature room. The solvent used was the mixture of 1,650 ml of sec-butanol containing 1.56g of trichloroacetic acid, 225 ml of propionic acid, and 1,305 ml of deionized water. In 80 ml of this solvent, 500mg of the globin was dissolved and placed in 4 tubes of the machine, that is, from tube No. 3 to 6. The upper phase transfer carried out 150 times by setting the machine for a 30 second shifting time and a 20 minute separating time. The solution in each tube after cleared with 0.5 ml of 50% ethanol was analyzed by absorbance at 280 μ. Solution at peaks was collected respectively, dialyzed against deionized water, and dried by lyophilization.

4) Aminoethylation of the polypeptide chain

Aminoethylation was carried out according to a modification of the method of JONES. The solvent used was Tris-EDTA buffer containing 8 M urea, which was prepared in the following way. Crystalline urea (36 g) and disodium ethylenediaminetetraacetate (150 mg) were dissolved in 30 ml of tris-buffer, pH 8.6 and to this was added deionized water so that the total volume was 75 ml. The tris-buffer pH 8.6 was made by dissolving 5.3 g of tris hydroxymethyl aminomethan in 1.0 N HCl and adding deionized water so that the whole volume was 30 ml. In 30 ml of the above-described solvent was dissolved 500 mg of the β polypeptide chain and then the solution, after aerated with N₂ gas for 15 minutes, was added 0.4 ml of the β-mercaptoethanol and allowed to stand at room temperature for 45 minutes in a sealed vessel. The mixture further added 0.9 ml of ethylene imine was allowed to react 45 minutes more and finally dialyzed against deionized water.

5) Digestion of aminoethylated β polypeptide chain with trypsin

Trypsin (Worthington Biochemical Corp., twice-crystallized) was dissolved in 1/16 N HCl to a concentration of 1% and the solution maintained at 37°C for 16 hours so that the chymotrypsin-like activity
was lost. In 50 ml of deionized water, 500 mg of the aminoethylated β polypeptide chain was suspended. The suspension adjusted to pH 9.0 with 0.1N NaOH was vigorously stirred at 37°C for 10 minutes.

Hydrolysis was carried out at 37°C for 4 hours by addition of 1 ml of the above-described trypsin solution (10 mg trypsin). The hydrolysate was readjusted to pH 4.0 with 1 N acetic acid and finally lyophilized.

6) Column chromatography of tryptic peptides

The resin (Dowex 1×2, 200-400 mesh) was washed with 1 N NH₄OH, water, acetic acid, and water. After suspended in the starting buffer and evacuated, it was poured into a column (2.0×150cm) warmed at 37°C, and then equilibrated with 4 1 of the starting buffer.

Tryptic peptides (500 mg, the β chain) dissolved in 30 ml of deionized water and brought to pH 10.0 by adding 0.1 N NaOH were applied on the column. Elution was carried out at a flow rate of 180 ml/hr by warming the column at 37°C and the effluent was collected in 18 ml-fractions. Fractions No. 1 to 20 were developed with the starting buffer (1% pyridine 1% collidine acetate buffer, pH 9.0) and Fractions No. 21 to 40, with 1% pyridine 1% collidine acetate buffer, pH 8.4. Henceforth, the gradient elution with acetic acid was employed. Fractions No. 41 to 170 were eluted by supplying 0.08 N acetic acid and Fractions No. 170 to 250, by supplying 1.0 N acetic acid from the upper chamber to the mixing chamber containing 1,500ml of 1% pyridine, 2,4 lutidine, 1% α-picoline, acetate buffer pH 7.5.

After alkali hydrolysis, peptides were detected by ninhydrin reaction according to the method of YEMM and COCKING, that is, 0.2 ml portion of each fraction was transferred in a hard glass tube and to this was added 1.0 ml of 2.5 N NaOH, and then hydrolysis was carried out at 95°C for 2.5 hours in an oil bath. The hydrolysate, after cooled, was added 1.0 ml of 30% acetic acid, 0.5 ml of 0.2 M citrate buffer, pH 5.0, and 1.2 ml of ninhydrin-KCN solution and heated at 100°C for 15 minutes. The solution after cooled and diluted with 3 ml of 60% ethanol was analyzed by absorbance at 570μm. Ninhydrin-positive fractions were collected by the peak, dried under reduced pressure below 37°C, and dissolved in 5 ml of deionized water.

7) Paper chromatography of the tryptic peptides

The sample, which was isolated by the above-described column chromatography and dissolved in 5 ml of deionized water, was applied 50μl per spot on a sheet of Toyo filer paper No. 50. Development was carried out at 25°C in the constant temperature room by employing the descending method with the upper phase of the mixture of n-butanol, acetic acid, and water (4:1:5). In order to locate peptides on the paper, 0.2% ninhydrin-n-butanol solution was sprayed, and the paper was heated to color with an iron. For detection of peptides containing
tryptophan, arginine, histidine, and tyrosine, EHRLICH reaction,\textsuperscript{17} SAKAGUCHI reaction,\textsuperscript{9} PAULI reaction,\textsuperscript{18} and $\alpha$-nitrosnaphtol reaction\textsuperscript{12} were performed respectively on the paper chromatogram. In the case of purification, peptides located with 0.02\% ninhydrin-n-butanol solution were eluted with 5\% acetic acid.

8) \textit{Paper electrophoresis of peptides}

The sample was applied on a line 18 cm from the anode side on a sheet of Whatman 3 MM (55 x 55 cm). Electrophoresis was carried out at 1.5 KV for 3.5 hours by hanging method in the pyridine acetate buffer, pH 6.4 (pyridine, acetic acid, water; 100 : 4 : 900). Peptides were located and eluted in the same way as in the case of the paper chromatography.

9) \textit{Amino acid analyses of the tryptic peptides}

The purified peptides were dissolved in 4 ml of constant boiling point HCl (twice-distilled) and hydrolyzed in sealed tubes at 105°C for 24 or 48 hours. The hydrolysates after dried were subjected to an amino acid analyzer, Hitachi KLA-2. The presence of tryptophan was confirmed by EHRLICH reaction.

\textbf{RESULTS AND DISCUSSION}

Hemoglobin from the hemolysate of adult Japanese monkeys (\textit{macaca fuscata fuscata}) was removed heme in HCl-acetone solution so that globin was obtained. The globin was separated into $\alpha$ and $\beta$ polypeptide chains by countercurrent distribution. The result is shown in Fig. 1.

![Countercurrent distribution of the globin from Japanese monkey hemoglobin](image_url)
From 500 mg of the globin, 160 mg of the \( \alpha \) polypeptide chain and 180 mg of the \( \beta \) polypeptide chain were obtained. The purity of the \( \beta \) polypeptide chain was known to be over 90% from the result of the N-terminal analysis by the DNP method\(^{19}\). The \( \beta \) polypeptide chain was aminoethylated by the modification of the method of JONES\(^{10}\). He used column chromatography with Sephadex G25 in order to remove various reagents used in the aminoethylation. Instead, dialysis against deionized water was carried out in this study. It turned out to be quite satisfactory. The \( \beta \) polypeptide chain thus aminoethylated came to be precipitated in the dialyzing sac.

The aminoethylated \( \beta \) polypeptide chain was hydrolyzed with trypsin at 37°C at pH 8.3. The enzyme concentration against the substrate was 2%. The hydrolysate which was at first turbid became almost clear 1 hour after the addition of trypsin, however, hydrolysis was continued for 4 hours. It is said that hemoglobins from various kinds of animals contain the so-called "core" which is precipitated when the pH of the tryptic peptides of both \( \alpha \) and \( \beta \) polypeptide chains is brought to 6.4. Therefore, the pH of the hydrolysate was gradually lowered to 4.0 by the addition of 1 N acetic acid. However, within this range of pH value, turbidity could not be recognized, suggesting "core" turned soluble because cysteine contained in it was aminoethylated.

Isolation of the tryptic peptides was performed by column chromatography of Dowex 1×2 with acetate buffer containing organic bases such as pyridine, collidine, picoline, and lutidine. The results are given in Fig. 2.

![Column chromatography of the tryptic peptides from the aminoethylated \( \beta \) polypeptide chain of Japanese monkey hemoglobin](image-url)
In the chromatography of this system, the pH of the starting buffer used generally is about 8.5, however in this experiment, development started with the starting buffer, pH 9.0 aiming at good isolation of peptides of the basic group. It is sure that the buffer of this system gave satisfactory results in isolation of peptides of the basic group, though the buffer action was reduced near pH 9.0.

In order to examine the purity of the peptides contained in peaks fractionated by the column chromatography, descending paper chromatography was performed with the upper phase of the system of n-butanol, acetic acid, and water (4:1:5). The resulting chromatogram is shown in Fig. 3. As shown in Fig. 3, though some are purely isolated only by the column chromatography, most peaks contained more than two peptides, which needed to be further purified by paper chromatography with the same system. The peptides thus isolated were hydrolyzed in constant boiling point HCl at 105°C for 24 or 48 hours, and then the amino acid compositions were analyzed. The results are given in Table I. The values were not corrected the losses during the hydrolysis and are given by molar ratios.

Identification and quantitative analysis of the aminoethyl cysteine were performed by using the sample obtained from Cyclo Chemical Corporation, Los Angeles, Calif., USA, as the standard for the amino acid analyzer. As a result, aminoethyl cysteine was eluted with 38 ml of pH 5.28 buffer on a 7.5 cm column. It was also analyzed after HCl hydrolysis and another peak was eluted with 35 ml of pH 3.25 buffer on a 50 cm column. It appeared to be due to cysteine. As for trypto-
Table I

Amino acid compositions of the tryptide peptides from the amino-ethylated β polypeptide chain of Japanese monkey homoglobin

<table>
<thead>
<tr>
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<th>IIIc</th>
<th>IVb</th>
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Yield (%): 45 28 40 22 59 32 27 31 29 15 34 37 49 66 48

phan, peptides positive to EHRlich reaction on the paper chromatogram are given (+) in Table I. The values in % given in the lowest column of the Table are the yields of peptides through all the procedures from aminoethylation to isolation and purification, which were calculated regarding the molecular weight of the β polypeptide chain as 16,000.

Concerning the peptides obtained from each spot on the paper chromatogram shown in Fig.3, the following discussion are made in comparison with the tryptic peptides from the β polypeptide chain of human hemoglobin (HβT)9,10 and of rhesus monkey (macaca mulatta) hemoglobin (MMβT)10,11 which have already been known.

Ia The amino acid composition, which are not shown in Table I, was Lys, 2.00; His, 0.92; Gly, 1.03; Ala, 1.05. The yield was 36%. This peptide appears to be a combination of IIa and IIIa, and quite similar to HβT7–8 and MMβT 7–8.

Ib The results from both paper chromatography and amino acid analysis confirmed it was arginine, however, the yield was so low as 3%.

IIa As shown in Table I, only lysine was a component of this fragment, which was also detected at the same position with lysine on the paper chromatogram, suggesting that it corresponded to βT8.
The amino acid composition was quite the same with those of HβT14 and MMBT14. The value of valine was 2.23 after 24 hour HCl hydrolysis and 2.65 even after 48 hour hydrolysis as shown in Table I. They seem to be low, considering that 3 moles of valine existed in this peptide. This fact suggests that there existed Val-Val bond in this peptide and showed strong resistance against HCl hydrolysis.

The composition was quite the same with those of HβT7 and MMBT7. It appears to have the same amino acid sequence with the basic center peptide (Ala-His-Gly-Lys) which are found in the β polypeptide chain from hemoglobins of most animals.

The composition was, as given in Table I, quite similar to that of the octapeptide from the C-terminus of HβT12 or MMBT12, a part of the so-called "core", whose sequence is Leu-Leu-Gly-Asp (NH₂)-Val-Leu-Val-CysH-Val-Leu-Ala-His-His-Phe-Gly-Lys. It corresponds to what JONES[10] called βT12-b which was considered to be produced because cysteine was aminoethylated and so the peptide bond in the C-terminal side of cysteine was cleaved with trypsin.

It is quite the same with HβT6 and MMBT6, Val-Lys.

The composition after 24 hour hydrolysis was Lys, 1.02; His, 1.98; Asp, 0.99; Gly, 1.06; Ala, 3.92; Val, 2.34; Leu, 1.08; Tyr, 0.69. The yield was 21%. This is a combination of HβT14 and VIIIa, namely, βT 14–15.

It was positive to tryptophan reaction. The composition shown in Table I is quite similar to that of MMBT2, but IVb has aspartic acid and threonine one mole more and serine and alanine one mole less than HβT2.

The ninhydrin color reaction on the paper chromatogram suggested that it contained two peptides. However, since the color reactions for tryptophan, tyrosine, and arginine were positive all over the spot, it was first analyzed as a single peptide. The composition was Arg, 0.341 μmole; Asp, 0.268 μmole; Thr, 0.325 μmole Glu, 0.392 μmole; Pro, 3.222 μmoles; Gly, 0.286 μmole; Val, 0.878 μmole; Leu, 1.432 μmoles; Tyr, 0.290 μmole; aminoethyl Cys, 0.251 μmole. This result clearly shows it is a mixture of more than two peptides. Therefore, the eluate from this spot was further purified by high voltage paper electrophoresis. Consequently, it was separated into two spots, IVc-1 negative to the color reactions for tryptophan, tyrosine, and arginine, and IVc-2 positive to all the color reactions mentioned above.

The composition was as shown in Table I, the same with the octapeptide of HβT12 or MMBT12 from the N-terminus to cystein residue.
This peptide should be, therefore, designated MF\(\beta\)T12-a.

**IVc-2** The amino acid composition after 48 hour hydrolysis is shown in Table I. It is quite similar to that of H\(\beta\)T4, but IVc-2 has valine one mole more and leucine one mole less than MM\(\beta\)T4.

**Va** The composition was Lys, 1.04; His, 0.96; Asp, 1.03; Thr, 1.01; Ser, 1.02; Glu, 1.92; Gly, 1.05; Ala, 1.03; Leu, 1.81; Phe, 0.93; AE Cys. The yield was 8%. It is quite the same with that of MM\(\beta\)T10, but Va has glutamic acid one mole more and threonine one mole less than H\(\beta\)T10.

**Vb** The composition is as given in Table I. It is quite similar to that of MM\(\beta\)T13, but Vb has glutamic acid one mole more and proline one mole less than H\(\beta\)T13.

**VIIa** It is, as shown in Table I, a peptide consisted of 22 amino acid residues, 2 moles of which are lysine. There exists a peptide designated \(\beta\)T10–11 in the so-called "core" insoluble at pH 6.4 of the tryptic peptides from the \(\beta\) chain of human hemoglobin. It is said that this combination is caused since the existence of aspartic acid residue preceded the C-terminal lysine makes the bond unsusceptible to trypsin. VIIa has glutamic acid and lysine one mole more and threonine and arginine one mole less than H\(\beta\)T10–11 but it is quite similar to MM\(\beta\)T10–11 in the composition. In this experiment, as mentioned previously, the peptide corresponding to \(\beta\)T10 was recovered as Va whose yield was 8%, however, the peptide corresponding to \(\beta\)T11 recovered so small in amount that it could not be confirmed. This fact suggests that the linkage between \(\beta\)T10 and \(\beta\)T11 won't cleave with trypsin even after aminoethylation. It is also considered that as Jones pointed out, unlike the cystein in \(\beta\)T12, the cystein in \(\beta\)T10 is not digested with trypsin even after aminoethylation.

**VIIIa** It had the same composition with H\(\beta\)T15 or MM\(\beta\)T15, Tyr-His.

**IXa** It had the same composition with MM\(\beta\)T9, but had aspartic acid one mole more and alanine one mole less than H\(\beta\)T9.

**Xa** It is quite the same with H\(\beta\)T1 and MM\(\beta\)T1 in their compositions.

**XIa** The amino acid composition was Lys, 2.03; His, 1.95; CysO\(_3\), 0.93; Asp, 2.89; Thr, 0.91; Ser, 0.88; Glu, 3.09; Pro, 1.05; Gly, 1.09; Ala, 1.12; Val, 0.95; Leu, 3.12; Phe, 1.93. The yield was 8%. The above composition is quite the same that of VIIa except that unaminoethylated cystein was supposed to change into cysteic acid by oxidation during the procedure.

**XIIa** The amino acid composition was quite similar to that of MM\(\beta\)T3
or H\(\beta\)T3. This peptide was completely purified only by column chromatography and the yield was pretty high as 66%.

\(XIIIa\), \(XIIIb\) The composition of \(XIIIa\) plus \(XIIIb\) was similar to that of MM\(\beta\)T5, but it had serine one mole more and threonine one mole less than H\(\beta\)T5. Both \(XIIIa\) and \(XIIIb\) had the similar composition to each other, indicating that a peptide revealed itself as two spots because of the change of methionine during the procedure.

Table II summarizes comparisons of the amino acid compositions of the tryptic peptides from the \(\beta\) polypeptide chain of human (H\(\beta\)T) and rhesus monkey hemoglobin (MM\(\beta\)T) with those from the aminomethylated \(\beta\) polypeptide chain of Japanese monkey hemoglobin (MF\(\beta\)T).

The \(\beta\) polypeptide chain of Japanese monkey hemoglobin is composed of 146 amino acid residues just like that of most mammals. By this study, however, it is presumed that Japanese monkey hemoglobin is different in seven amino acid residues from human hemoglobin, that is, Asp\(\rightarrow\)Ser, Thr\(\rightarrow\)Ala, Ser\(\rightarrow\)The, Ala\(\rightarrow\)Asp, Glu\(\rightarrow\)Thr, Lys\(\rightarrow\)Arg, and Glu\(\rightarrow\)Pro. In addition, it differs in one amino acid residue in \(\beta\)T4 peptide from rhesus monkey hemoglobin which belongs to Japanese monkey hemoglobin.

**Table II**

Comparison of the amino acid compositions of the tryptic peptides from the \(\beta\) polypeptide chain of Japanese monkey, rhesus monkey and human hemoglobins

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<thead>
<tr>
<th>Peptide</th>
<th>(\beta)T1 FMH</th>
<th>(\beta)T2 FMH</th>
<th>(\beta)T3 FMH</th>
<th>(\beta)T4 FMH</th>
<th>(\beta)T5 FMH</th>
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<th>(\beta)T7 FMH</th>
<th>(\beta)T8 FMH</th>
<th>(\beta)T9 FMH</th>
<th>(\beta)T10-11 FMH</th>
<th>(\beta)T12a FMH</th>
<th>(\beta)T12b FMH</th>
<th>(\beta)T13 FMH</th>
<th>(\beta)T14 FMH</th>
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the same genus with Japanese monkeys. It seems an exchange between valine and leucine.

CONCLUSION

The β polypeptide chain of adult Japanese monkey (*macaca fuscata fuscata*) hemoglobin after aminoethylation was hydrolyzed with trypsin. Tryptic peptides obtained were isolated and purified by column chromatography, paper chromatography, and high voltage paper electrophoresis, and then their amino acid compositions were analyzed. The results were compared with the corresponding data from human and rhesus monkey hemoglobins. It is presumed that Japanese monkey hemoglobin differs in seven amino acids of five peptides from the β polypeptide chain from human hemoglobin, and also differs in only one amino acid from rhesus monkey hemoglobin which belongs to the same genus.

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REFERENCES