Amino Acid Substitution in Hemoglobin M_{Akita}

Takaoki MIYAJI, Iwao IUCHI,
Kosaku KARITA, Yuzo OHBA,
Kiyomi YAMAMOTO and
Susumu SHIBATA
3rd Division, Department of Internal Medicine, Yamaguchi University School of Medicine
(Received May 31, 1967)

Hemoglobin surveys[1] carried out in several districts for the recent ten years have disclosed the presence of a great variety of abnormal hemoglobins distributed among the Japanese despite rarity of hemoglobinopathy in Japan. No less than 28 variants of hemoglobins have been detected. Hemoglobin M's are the most note-worthy of them all, since they are associated with a characteristic clinical manifestation, that is cyanosis.

It was in 1960 that Hb M_{Iwate} was discovered, for the first time, in a patient with hereditary nigremia (Tamura-Takahashi's disease), which had been an endemic disease of unknown etiology in Iwate Prefecture for about 160 years.[2] In the same year Hb M_{Kurume} (identical with Hb $M_{Saskatoon}$) was detected in a cyanotic boy suspected of congenital heart disease in Kurume.[3] Four years later, in 1964, Hb M_{Osaka} (identical with Hb M_{Boston}) was encountered in Osaka.[4]

Hemoglobin has a molecule composed of two sorts of polypeptide chains, the α and the β, united in a manner expressed by $\alpha_2\beta_2$. Each chain has two functionally important histidine residues, the proximal (α87, β92) and the distal (α58, β63). The proximal histidine acts the role of holder of heme iron, securing to fix the heme to the relevant chain, while the distal histidine prevents heme iron from autoxidation, enabling hemoglobin molecule to combine with oxygen molecule reversibly for the purpose of transportation.[5]

Studies on the primary structure of the abnormal polypeptide chains of Hb M's of the Japanese have revealed that the proximal histidine of the α chain is replaced by tyrosine in Hb M_{Iwate},[6] and the distal histidine of the same chain is substituted for by tyrosine in Hb M_{Osaka}.[7] In Hb M_{Kurume} tyrosine is in substitution for the distal histidine of the β chain.[8] Accordingly, it was presumed by analogy from the relationship of Hb M_{Iwate} (α proximal histidine anomaly) to Hb M_{Osaka} (α distal histidine anomaly) that a new Hb M possesing tyrosine in place of proximal histidine of the β chain would be encountered in the future as the

This investigation was supported in part by a PHS Research grant (GM 09469-05) from the Division of General Medical Sciences, U.S. Public Health Service and a grant from the Ministry of Education of Japanese Government.

counterpart of Hb M_{Kurume} (β distal histidine anomaly). Nevertheless, such a varient of Hb M has never been found anywhere throughout the world until quite recent days.

In May, 1966, the hemoglobin survey laboratory in Ube received a blood sample collected from a patient with congenital cyanosis living in Akita Prefecture. Since Akita is adjacently situated to Iwate, where Hb M_{Iwate} disease is distributed, the same disease was naturally conceived as a possible diagnosis for this patient. Conventional techniques for the detection of Hb M successfully demonstrated the presence of this kind of abnormal hemoglobin in patient's blood. However, scrutiny of the results of examinations unravelled that the hemoglobin did not conform in properties with any one of Hb M's so far recorded in Japan. In addition, further study by chemical analysis has established that this is indeed the Hb M whose proximal histidine of the β chain is substituted for by tyrosine. The hemoglobin was named Hb M_{Akita}, because it was thought to be the new variant of Hb M, the existence of which had been conceived by us for these several years.[9]

This paper aims to report the results of our study on the primary structure of Hb M_{Akita}.

MATERIALS AND METHODS

1. Demonstration of Hb M_{Akita}[10]: — Patient's blood which was prevented from coagulation by addition of ACD solution was centrifuged to separate erythrocytes from plasma. Hemolysate was prepared from the erythrocytes by conventional techniques, and it was subjected as it was (O_2 Hb type) or after treatment with ferricyanide (metHb type) to absorption spectrophotometry, agar gel electrophoresis (pH 7.0 and 8.6) and Amberlite IRC 50 chromatography.

2. Demonstration of abnormal polypeptide chain[11]: — Solution of metHb M_{Akita} which was purified by starch block electrophoresis (see below) was reduced by dithionite, dialysed against phosphate buffer solution (M/100) in a Visking tube to remove dithionite, concentrated with Sephadex G200 and mixed with an appropriate solution of canine hemoglobin. Hybridization by dissociation and recombination, and demonstration of hybrid hemoglobins were carried out by the method described by Shibata et al.[11]

3. Purification of Hb M_{Akita}: — a) Hb M_{Akita}. metHb M_{Akita} was purified from metHb type hemolysate of the patient by starch block electrophoresis (pH 7.0). b) Mixture of β chains (β chain of Hb M_{Akita} + β chain of normal adult hemoglobin) which is free from α chain. O_2Hb type hemolysate of the patient was treated by Anson-Mirsky's technique[12] to get globins from Hb M_{Akita} and Hb A contained therein. CMC (carboxymethyl cellulose) column chromatography in 8M urea[13] was employed to prepare the β chains (α chain free) from the

globins. c) β chain. α chain-free β chain was obtained from the O_2Hb type hemolysate of normal subjects in the same way as described in b).

Hb M_{Akita}, and the mixture of β chains were examined by fingerprinting and amino acid analysis with the normal β chain as control.

4. Removal of heme from globins and β chain: — Anson-Mirsky[12] was followed.

5. Tryptic digestion of globins and β chains: — Ingram[14] was followed.

6. Fingerprinting of the tryptic digests: — Ingram's procedure[14] was used.

7. Aminoethylation of the globins and the β chains: — Jones' technique[15] was employed.

8. Fingerprinting of the tryptic digests of the aminoethylated globins of β chains: — Ingram[14] was followed for the most part, but electrophoresis of longer duration with higher voltage (2500 V, 100 mA, for 3 to 4 hours) was used so that complete separation of neutral tryptic peptides might be achieved.

9. Detection of abnormal peptide spot on the fingerprint[16]: — Sprays with ninhydrin (for detection of abnormal peptide spots), α nitroso-β naphthol solution (for detection of tyrosine) and Pauly's solution (for detection of histidine).[17] were used.

10. Elution and acid hydrolysis of the abnormal peptide spots appeared on the fingerprint: — These were carried out by conventional techniques. The acid hydrolysis (with 6N HCl) was carried out at 105°C for 22 hours.

11. Amino acid analysis of the acid hydrolysate[18]: — an automatic amino acid analyzer of Yanagimoto Company was employed.

RESULTS

Methemoglobin M_{Akita} migrated to the anode side of metHb A, forming a clearly separated chocolate-brown stripe, when methemoglobin type hemolysate of the patient was subjected to agar gel electrophoresis (pH 7.0) (Figure 1～3).

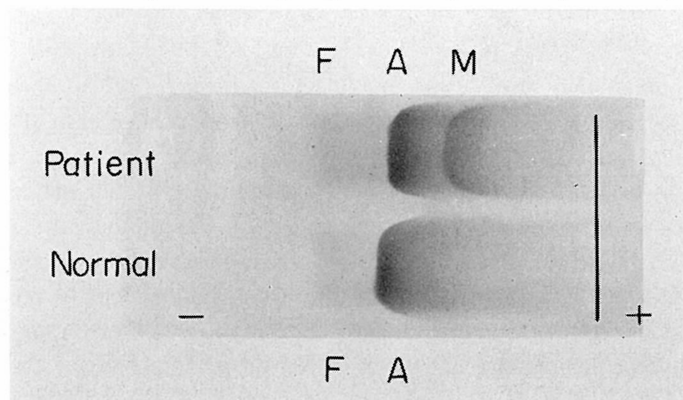

Fig. 1. Agar gel electrophoresis (pH 7.0) of oxyhemoglobin type hemolysate.
F: HbF, A: HbA, M: Hb M_{Akita}.

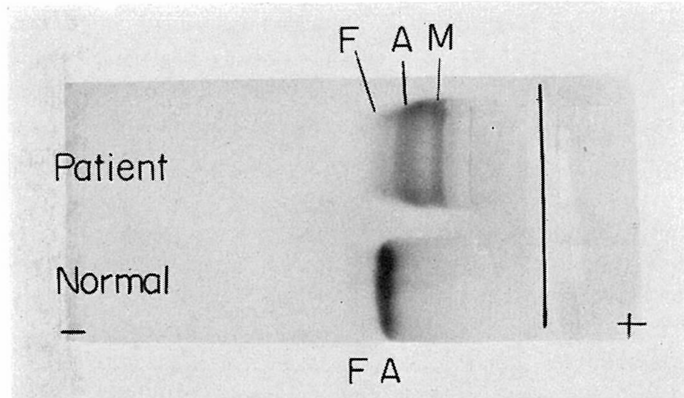

Fig. 2. Agar gel electrophoresis (pH 7.0) of methemoglobin type hemolysate. F: met Hb F, A: met Hb A, M: met Hb M_{Akita}.

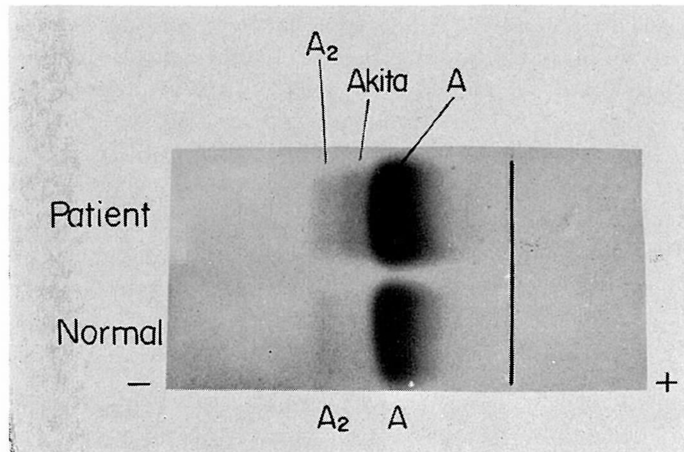

Fig. 3. Agar gel electrophoresis (pH 8.6) of methemoglobin type hemolysate. A_2: metHb A_2, Akita: metHb Akita (minor abnormal hemoglobin component), A: metHb A.

This component was also demonstrable by Amberlite IRC 50 chromatography, in which three layers of hemoglobins were seen on the column (Figure 4). Hb M_{Akita} was separated at the top as a gray-black layer, next came the dark red layer of Hb A and at the bottom lay the light red layer of the minor component. Hb F descended the column fast, going ahead of the minor compoenent.

This reddish hemoglobin relevant to the minor component was called Hb Akita in order to discriminate it from Hb M_{Akita} which had chocolate brown color.

Hybridization test with canine hemoglobin disclosed β chain anomaly in Hb M_{Akita} (the hybrid hemoglobin $\alpha_2^{Can}\beta_2^{M}$ migrated to the anode faster than the hybrid hemoglobin $\alpha_2^{Can}\beta_2^{A}$) (Figure 5). Yet, the fingerprint of the tryptic digest of the globin was not consistent with it. The pattern of the peptide spots was normal without any spots of unusual appearance.

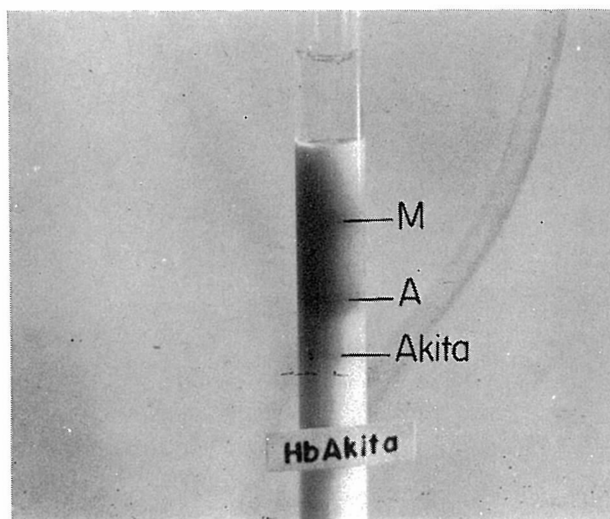

Fig. 4. Amberlite IRC 50 chromatography of oxyhemoglobin type hemolysate of the patient.
Akita : Hb Akita (minor abnormal hemoglobin component, red),
A : HbA (red), M : Hb M_{Akita} (gray-black).

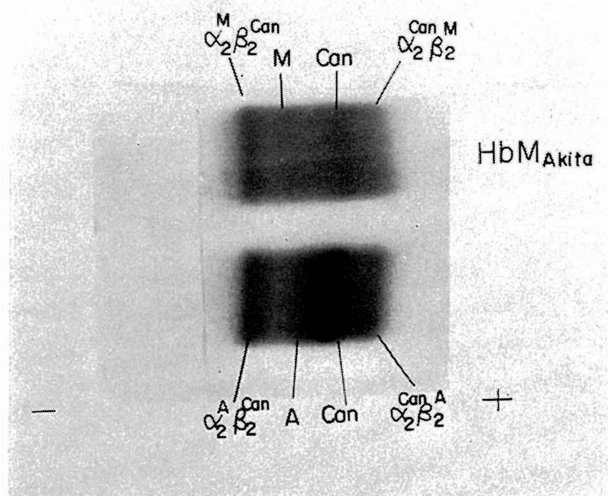

Fig. 5. Starch gel electrophoresis (pH 8.6) of hybrid hemoglobins. Hybrid hemoglobin $\alpha_2^{Can}\beta_2^M$ (which is composed of the β chain of Hb M_{Akita}) is slightly faster than the corresponding hybrid hemoglobin from the normal β chain of Hb A ($\alpha_2^{Can}\beta_2^A$) in anodal migration.

Abnormal peptide spots were visualized only when the tryptic digest of aminoethylated globin of Hb M_{Akita} was fingerprinted (Figure 6). Two spots were seen: spot (a) lying in the cathodal vicinity of a spot of peptide which was identical in amino acid composition with βTp-13 (Glu 121～Lys 132), and spot (b) sitting just below the same peptide spot (Figure 6). Incidentally, a peptide spot of the amino acid composition compatible with that of βTp-11 (leu 96～Arg 104) was also located right above the peptide spot βTp-1 (Val 1～Lys 8). Distincter appearance of the spots (a) and (b) was achieved with the tryptic digest of the aminoethylated β chain mixture ($\beta^M+\beta^A$) (Figure 7).

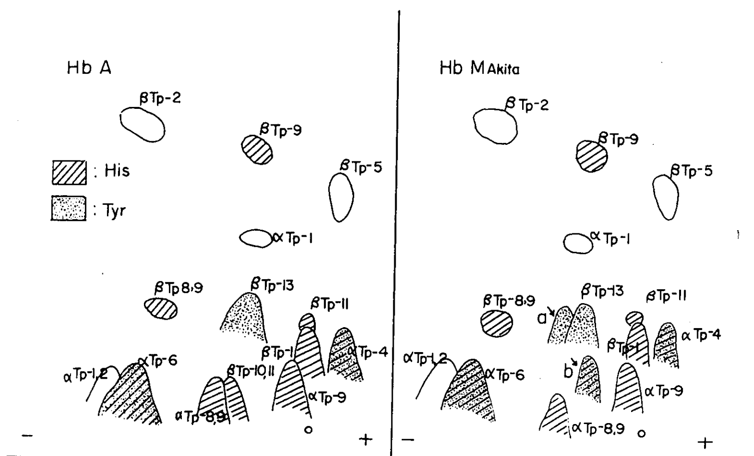

Fig. 6. Schematic representation of the fingerprint of the tryptic digest of the aminoethylated whole globin of Hb A (left) and Hb M_{Akita} (right). Arrows indicate the abnormal spots a and b. Note the absence of the β Tp-10, 11 spot on the Hb M_{Akita} fingerprint in spite of the presence of thc β Tp-11 spot in the proper place. On the Hb A fingerprint, the β Tp-10 spot is hidden in the αTp-6 spot.

Therefore, the abnormal tryptic peptides (a) and (b) were eluted from the fingerprint of the β chain mixture for the purpose of the analysis of their amino acid composition.

The results showed that the spots (a) and (b) were in good agreement with the tryptic peptides βTp-10 and βTp-10, 11 of the normal adult hemoglobin, respectively.

Amino acid analysis showed that both spots (a) and (b) possessed tyrosine and aminoethylated cysteine. Spot (a) lacked histidine residue and spot (b) had only one residue of histidine. Apart from this characteristic feature, these spots were in good agreement in amino acid composition with the tryptic peptides βTp-10 and βTp-10, 11 of Hb A (normal adult hemoglobin) (Table 1).

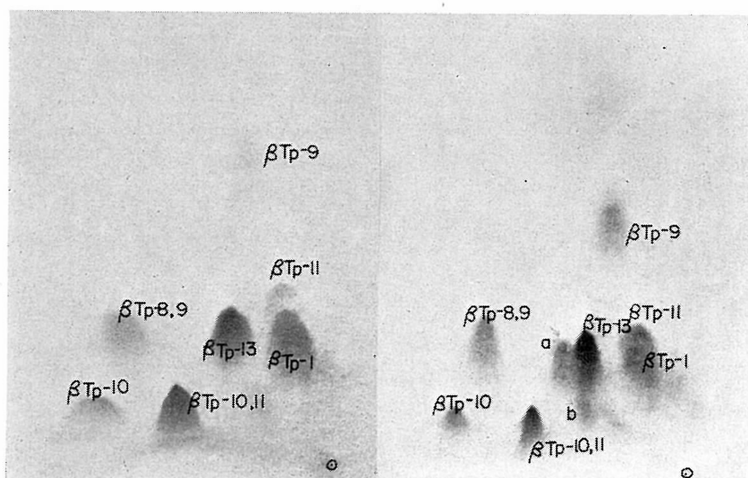

Fig. 7. Fingerprint of the tryptic digest of the aminoethylated β chain from the normal adult hemolysate (left) and from the patient's hemolysate (right); the latter is composed of the mixture of the normal and Hb M_{Akita} β chain. Distincter visualization of the abnormal spots a and b is achieved.

Table 1. Amino acid analysis of the abnormal peptides (a and b) together with that of peptide βTp-11.

	Lys	Arg	His	Ala	Gly	Ser	Thr	Pro	Val	Phe	Leu	Cyst	Tyr	Glu	Asp	Total
βTp-10 Theoretical	1		1	1	1	1	2			1	2	1		1	1	13
abnormal spot a actual	0.88		0.18	1.23	0.52	0.98	1.74			1.17	1.89	+	0.67	1.05	0.88	
estimated	1		0	1	1	1	2			1	2	+	1	1	1	13
βTp-10・11 Theoretical	1	1	2	1	1	1	2	1	1	2	3	1		2	3	22
abnormal spot b actual	1.20	0.92	0.91	0.98	0.85	1.13	1.76	0.92	1.02	2.13	2.40	+	0.78	2.24	2.51	
estimated	1	1	1	1	1	1	2	1	1	2	3	+	1	2	3	22
βTp-11 Theoretical		1	1					1	1	1	1			1	2	9
spot βTp-11 actual	0.09	0.88	0.88	0.04	0.07	0.06	0.07	0.97	1.06	1.19	0.77			1.06	2.29	
estimated	0	1	1	0	0	0	0	1	1	1	1			1	2	9

DISCUSSION

It is apparent from the afore-going account of the results of electrophoresis and hybridization test that Hb M_{Akita} is a hemoglobin of β chain anomaly possessing an amino acid substitution prone to result in increase in the negative

charge of the relevant chain.

Hemoglobin M_{Akita} forms about 30 per cent of the total hemoglobins contained in patient's hemolysate, but there is a certain degree of difficulty in obtaining it in sufficient amount by starch block electrophoresis, because it produces a considerable trail of deteriorated precipitate. Consequently, CMC column chromatography in 8M urea of the globins (of both Hb M_{Akita} and Hb A) prepared from patient's hemolysate was resorted to in order to get the raw material for the collection of abnormal peptides by fingerprinting.

The fact that the fingerprint of Hb M_{Akita} without pretreatment by aminoethylation is normal in pattern attracts our special attention, since it makes us presume that the amino acid substitution of its β chain is not localized in the part generative of soluble tryptic peptides (β Tp-1, 2, 3, ..., 9, 13, 14 and 15), but concealed in the core (β Tp-10, 11 and 12 or Gly 83 ~ Lys120).

The presumption has been corroborated by fingerprinting of the aminoethylation. Abnormal spots (a) and (b) are the evidence.

The composition of these peptides is of particular interest. As mentioned previously they resemble closely peptides β Tp-10 and β Tp-10, 11 in the core, but both of them contain tyrosine which is not a proper constituent of β Tp-10 and β Tp-10, 11. Tyrosine is therefore thought to be involved in the amino acid substitution. HbM_{Akita} is a fast-moving hemoglobin which has a negative charge larger than that of Hb A. The amino acid residue likely to be replaced by tyrosine in this hemoglobin is supposed to be histidine, lysine or arginine, since substitution of tyrosine for these basic amino acid residues makes the only one possibility of giving rise to a fast-moving hemoglobin.

That a spot pertaining to β Tp-11 is seen in its proper place on the fingerprint of aminoethylated globin of Hb M_{Akita} is a note-worthy finding, because this provides evidence for the normal composition of its β Tp-11 notwithstanding that its β Tp-10, 11 (i. e. β Tp-10 + β Tp-11) is really abnormal. It is accordingly concluded that in Hb M_{Akita} the amino acid substitution is present in β Tp-10.

The peptide β Tp-10 of Hb A has only one histidine residue at the ninety-second site (His β 92). In Hb M_{Akita} the abnormal peptide (a) has tyrosine (one residue) but no histidine in spite of its otherwise complete agreement with normal β Tp-10. This abnormal peptide is interpreted as a modification of normal β Tp-10, in which the only one histidine residue, His (β 92), is lost and Tyr (one residue) has been placed in stead of it in the same place. Hb M_{Akita} is therefore inferred to be an abnormal hemoglobin of β chain anomaly whose His (92 β) is substituted for by tyrosine. Since histidine is a basic amino acid, such a sort of amino acid substitution is consistent with the electrophoretic behavior of this abnormal hemoglobin.

Unfortunately, chemical study of the red minor component, Hb Akita, has not yet been accomplished. However, it is likely that this reddish hemoglobin is a

modification of the chocolate-brown hemoglobin, i e. Hb M_{Akita}.

Three variants of Hb M's have hitherto been recorded in Japan. They are Hb M_{Iwate} ($\alpha_2^{87Tyr}\beta_2$), Hb M_{Kurume} ($\alpha_2\beta_2^{63Tyr}$) and Hb M_{Osaka} ($\alpha_2^{58Tyr}\beta_2$). It is apparent that Hb M_{Akita} is a new hemoglobin that has never been encountered in this country, because it is $\alpha 2\beta_2^{92Tyr}$.

However, by perusal of literatures it has been learned recently that a hemoglobin which is the same in primary structure as Hb M_{Akita} was detected several months earlier than our discovery from a negro patient living in the United States and named Hb $M_{Hyde\ Park}$ by Heller and his associates.[19] Now, Hb M_{Akita} is the second example of Hb $M_{Hyde\ Park}$ so far known in the world.

SUMMARY AND CONCLUSION

A new variant of Hb M was discovered from a family of hereditary cyanosis living in Akita by combined use of conventional tests for Hb M including spectroscopy of methemoglobin type hemolysate, agar gel electrophoresis (pH 7.0) and Amberlite IRC 50 chromatography. The hemoglobin was called Hb M_{Akita}.

MetHb M_{Akita} was purified by starch block electrophoresis (pH 7.0) of the metHb hemolysate of patient and deprived of heme to get M_{Akita} globin. Mixture of β chains ($\beta^M+\beta^A$) was also purified by CMC chromatography in 8M urea of the globin (Hb M_{Akita} + Hb A) prepared from the patient's hemolysate.

The globin and the β chain mixture were aminoethylated, digested, with trypsin and then fingerprinted. The fingerprint thus made revealed abnormal spots related to peptides βTp-10 and βTp-10, 11. These were eluted, and hydrolyzed with hydrochloric acid. The amino acid analysis of the acid hydrolysate has demonstrated that, in Hb M_{Akita}, the ninety-second residue of the β chain, His (β 92), is substituted for by tyrosine. This hemoglobin is therefore expressed by the formula $\alpha_2\beta_2^{92Tyr}$. Hb M_{Akita} is identical with Hb $M_{Hyde\ Park}$ which was detected from a negro patient in the United States several months earlier.

REFERENCES

1) Shibata, S., Iuchi, I. and Miyaji, T.: Abnormal hemoglobins discovered in Japan. *Acta Haem. Jap.*, **29**: 115～127, 1966.

2) Shibata, S., Tamura, A., Iuchi, I. and Takahashi, H.: Hemoglobin M_I: Demonstration of a new abnormal hemoglobin in hereditary nigremia. *Acta Haem. Jap.*, **23**: 96～105, 1960.

3) Kimura, N., Nishimoto, S., Nawata, Y., Mori, F., Kodama, S. and Nakakura, S.: Hemoglobin M disease. A case report. *Jap. Heart J.*, **1**: 456～465, 1960.

4) Hayashi, A, Yamamura, Y., Ogita, Z., Ogita, S. and Kikkawa, H.: Hemoglobin M_{Osaka}, a new variant of hemoglobin M. *Jap. J. Human Genet.*, **9**: 87～94, 1964.

5) Lehmann, H., and Huntsman, R. G.: *Man's Hemoglobins.* North Holland (Amsterdam), 1966.
6) Shibata, S., Tamura, A., Iuchi, I. and Miyaji, T.: Hereditary nigremia and hemoglobin M_{Iwate}. *Proc. Jap. Acad.*, **40**: 220～225, 1964.
7) Shimizu, A., Hayashi, A., Yamamura, Y., Tsugita, A. and Kitayama, K.: The structural study on a new hemoglobin variant, Hb M_{Osaka}. *Biochim. Biophys. Acta*, **97**: 472～482, 1965.
8) Shibata, S., Miyaji, T., Iuchi, I., Ueda, S., Takeda, I, Kimura, N. and Kodama, S.: Hemoglobin M_{Kurume}: Its identity with hemoglobin $M_{Saskatoon}$. *Acta Haem. Jap.*, **25**: 690～694, 1962.
9) Shibata, S, Miyaji, T., Karita, K., Iuchi, I., Ohba, Y. and Yamamoto, K.: A new type of hereditary nigremia discovered in Akita — Hemoglobin $M_{Hyde\ Park}$ disease. *Proc. Jap. Acad.*, **43**: 65～70, 1967.
10) Shibata, S., Iuchi, I., Miyaji, T. and Ueda, S.: Spectroscopic chracterization of Hb M_{Iwate} and Hb M_{Kurume}, the two variants of hemoglobin M found in Japan. *Acta Haem. Jap.*, **24**: 577～485, 1961.
11) Shibata, S., Iuchi, I., Ueda, S., Miyaji, T., and Takeda, I.: Agar gel electrophoresis of the hybrid of canine and human hemoglobins: A simple convenient means for the determination of chain anomaly. *Acta Haem. Jap.*, **25**: 675～681, 1962.
12) Anson, M. L. and Mirsky, A. E.: Protein coagulation and its reversal: the preparation of insoluble globin, soluble globin and heme. *J. Gen. Phys.*, **13**: 469～476, 1930.
13) Clegg, J. B., Naughton, M. A. and Weatherall, D. J.: Abnormal human hemoglobin. Separation and characterization of the α and β chain by chromatography, and the determination of two new variants, Hb Chesapeake and Hb $J_{Bangkok}$. *J. Mol. Biol.*, **19**: 91～108, 1966.
14) Ingram, V. M.: Abnormal hemoglobins. I. The comparison of normal human and sickle cell hemoglobins by "Fingerprinting". *Biochim. Biophys. Acta*, **28**: 539～545, 1958.
15) Jones, R. T.: Structural studies of aminoethylated hemoglobins by automatic peptide chromatography. *Cold Spring Habor Symposium on Quantitative Biology*, **24**: 297～308, 1964.
16) Shibata, S., Miyaji, T., Iuchi, I. and Tamura, A.: Substitution of tyrosine for histidine (87) in the α chain of hemoglobin M_{Iwate}. *Acta Haem. Jap.*, **27**: 13～18, 1964.
17) Feigl, F.: Spot Tests in Organic Analysis. Elsevier (Amsterdam, London, New York, Princeton), 1956.
18) Piez, K. A. and Morris, L.: A modified procedure for the automatic analysis of amino acids. *Anal. Biochem.*, **1**: 187～201, 1960.
19) Heller, P., Coleman, R. D. and Yakulis, V. J.: Structural studies of haemoglobin $M_{Hyde\ Park}$. The XI th Congress of the International Society of Haematology (Plenary Session), V. C. N Blight, Government Printer (Sydney), 1966 (pp. 427～437).