Hemoglobin F Ube (108 Asn→Lys ?), A New Abnormal Fetal Hemoglobin Found in a Japanese Baby

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For more than one hundred years, it has been known to the hematologist that new-born babies (or fetuses) have in their blood a peculiar hemoglobin, more resistant to alkali denaturation than the normal adult hemoglobin.[1] This alkali-resistant hemoglobin is characterized spectroscopically by a distinct tryptophan notch at 289 nm, owing to the amino acid (Try, Tyr and Phe). It makes a sharp contrast with hemoglobins (Hb $A_1=\alpha_2\beta_2$ and Hb $A_2=\alpha_2\delta_2$) which show obscure bending of the absorption curve (tryptophan inflection) at 291 nm.[2] Therefore, the human hemoglobin produced in the fetal stage of growth is called fetal hemoglobin (Hb F).

Hemoglobin is a chromoprotein of heme (prosthetic group capable of combining oxygen molecules reversibly) and globin (the protein portion carrying the prosthetic group). The latter is a tetramer of two pairs of polypeptide chains; the α and the non α, expressed as $(\alpha)_2$ (non $\alpha)_2$.[3] Hb A_1 ($\alpha_2\beta_2$), the major fraction of human adult hemoglobin, possesses an $\alpha\beta$ chain in its non-α polypeptide, while Hb A_2 ($\alpha_2\delta_2$) the minor fraction, has a δ-chain as its non α.[3] Hb F ($\alpha_2\gamma_2$) shares the same α chain with human adult hemoglobins, but it contains a unique non-α chain which is called the γ chain. The γ chain is a polypeptide with an amino acid sequence apparently different from those of β and δ chains.[3] In addition, recent study by Schroeder and his associates[4] revealed the presence of two types of γ chains, ($^G\gamma$ and $^A\gamma$) which can be distinguished by only one amino acid residue, i.e. the $^G\gamma$ chain contains Gly at the 136th site from the N-terminal, and the $^A\gamma$ chain contains Ala at the same site.

Evidence for the theory that the abnormal variants of human adult hemoglobins ($\alpha_2^X\beta_2$ or $\alpha_2\beta_2^Y$, where α^X and β^Y denote abnormal α or β chain) are the product of a one-point mutation of the amino acid sequence of the α or the β chain has been accumulated in abundance since 1959, when Ingram[5] successfully established the amino acid substitution of the abnormal β chain of the hemoglobin of sickle

cell anemia (Hb S). Similar examples supporting the view of a one point mutation of the γ chain are gradually increasing, although abnormal Hb F's ($\alpha_2\gamma_2^z$, where γ^z refers to the abnormal γ chain) are designated as abnormal fetal hemoglobins.

The abnormal fetal hemoglobins show the principal characteristic properties of normal Hb F, namely the strong resistance to alkali denaturation and the salient tryptophan notch. Gillespie (1959)[6] is credited with the presentation of the first instance of an abnormal fetal hemoglobin, and since then about 10 examples have been recorded over the world. In this country, two variants of abnormal fetal hemoglobin, Hb F Akashi[7][8] and Hb F Fukuoka[9] have been reported from Kyoto and Fukuoka.

In 1969, in the course of screening by agar gel electrophoresis the hemolysates prepared from the cord blood specimens collected in Ube, we found a hemoglobin stripe which migrated separately after Hb F to the anode at pH 8.6. We purified this slow moving hemoglobin in order to examine its light absorption in the ultraviolet range and its alkali denaturation. We analyzed its globin by fingerprinting and by amino acid analysis of the peptide spots visualized on the fingerprint map. On the basis of these examinations we presumed this hemoglobin to be a new abnormal fetal hemoglobin and named it Hb F Ube after the city where it was discovered.

The purpose of this paper is to present the result of our study made on this hemoglobin.

MATERIAL AND METHODS

Hemolysate was prepared from the cord blood and blood collected from the femoral vein of a male baby by the conventional technique,[3][10] and was subjected to agar gel (pH 8.6 and 7.0)[11] and cellulose acetate membrane (pH 8.6)[10] electrophoreses to detect abnormal hemoglobin. Portions of the stripe of abnormal hemoglobin which appeared on the cellulose acetate membranes were cut out, collected and eluted with an adequente amount of water to purify the solution.

The solution of purified abnormal hemoglobin thus obtained was examined for alkali denaturation by Jonxis's method,[12] and studied spectrophotometrically (effective band width 1 nm) over the ultraviolet range for the tryptophan notch. It was also treated by Anson Mirsky's procedure[13] to separate the globin from the abnormal hemoglobin by removing the heme.

The globin of the abnormal hemoglobin was digested with trypsin and fingerprinted by Baglioni's method.[14] The abnormal peptide spot appearing on the fingerprint map was eluted with 6 N HCl, and hydrolysed in a sealed glass tube at 105°C for 24 hours. The hydrolysate was analysed for amino acid composition by an automatic amino acid analyzer.[15]

The residue, or insoluble core, of the trypsin digest of the globin was collected and washed with distilled water (pH 6.4) several times, and suspended in about 20 ml of 0.1 N NH_4OH solution. The suspension was digested at 37°C for 4 hours with chymotrypsin (0.4 mg).[16] The digestion was stopped with addition of 1 N HCl. The digested material obtained in this manner was fingerprinted (Baglioni's method[14]). Its fingerprint map was compared with that of the core of the globin in normal Hb F, which has been treated in the same manner.

RESULTS

The abnormal hemoglobin that we detected in the cord blood was different from the normal human fetal hemoglobin (Hb F). In contrast to Hb F, the abnormal hemoglobin could not be distinguished from the normal human adult hemoglobin (Hb A) by agar gel electrophoresis at pH 7.0, but developed at pH 8.6 a somewhat indistinct stripe, extending cathode-wards from the main band, which was a mixture of Hb A_1 and Hb F. Its cathodic end did not touch the Hb A_2 stripe (Fig. 1). By cellulose acetate membrane electrophoresis at pH 8.6, the abnormal hemoglobin was ultimately separated from the main band (Hb A_1+Hb F) as a clearly delineated slow moving stripe (Fig. 2)

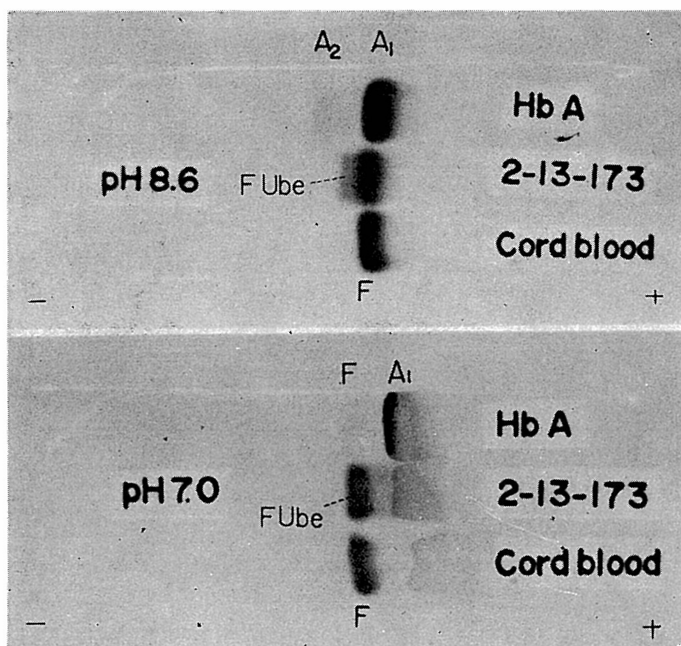

Fig. 1. Agar gel electrophoresis of hemolysate.

This technique (cellulose acetate membrane electrophoresis) enabled us to follow the changes in the level of the abnormal hemoglobin in the neonatal period. The

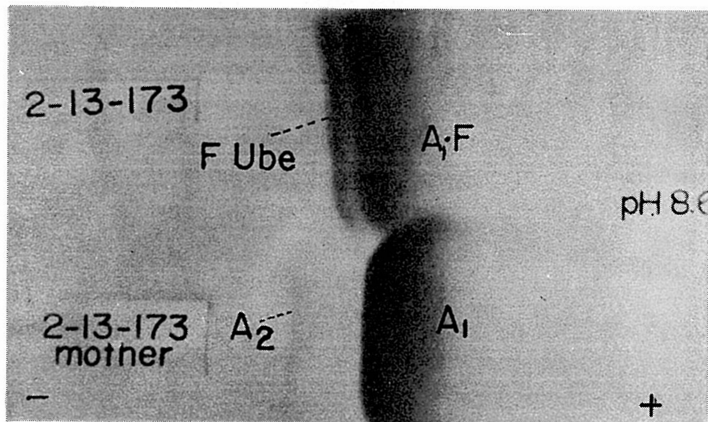

Fig. 2. Cellulose acetate membrane electrophoresis (pH 8.6) of hemolysate.
2-13-173 : Baby's hemlysate, 2-13-173 mother : Mother's hemolysate.

abnormal hemoglobin accounted for 15.5 per cent of the total amount of hemoglobin immediately after birth (cord blood) and remained at almost the same level for the subsequent 40 neonatal days. This level began to decrease on the 40th day, descending to 11.2 per cent, and disappeared completely on the 100th day. It deserves special mention that the content of Hb A_2 of the cord blood of this baby was apparently high (1.3 per cent of the total hemoglobin) in comparison with that found in the cord blood of ordinary babies (less than 0.3 per cent).

The solution of the purified abnormal hemoglobin exhibited a salient tryptophan notch (289 nm) in the ultraviolet region (Fig. 3). The same notch was also visible in the hemolysate prepared from the cord blood of the baby, although it was less distinct.

The abnormal hemoglobin was strongly resistant to alkali. The Jonxis method demonstrated that the optical density (576 nm) of its solution did decrease very slowly after addition of 1 N NaOH solution, but remained at a level as high as 78 per cent of the original even at the fifth second (Fig. 4).

Comparison of the fingerprint map of the globin in the abnormal hemoglobin with that of the normal hemoglobin (Fig. 5) revealed an extra peptide spot lying between the spot ♯14 (α Tp-10) and the spot ♯15 (γ Tp-6). Amino acid analysis of the hydrolysate of the eluate of this peptide spot indicated the proportion of Leu : Gly : Lys to be 0.33 : 0.54 : 1.00 = 1 : 1 : 1.

Analysis indicated that the peptides of other spots (including ♯7 = γ Tp-2, and ♯5 = γ Tp-9) were within the range of the corresponding spots of the normal Hb F fingerprint. The chymotrypsin digest of the core gave too many spots to enable us to identify individual peptides.

The baby possessing this abnormal hemoglobin was normal in physical examination, and no abnormality was demonstrable by classical routine hematological tests.

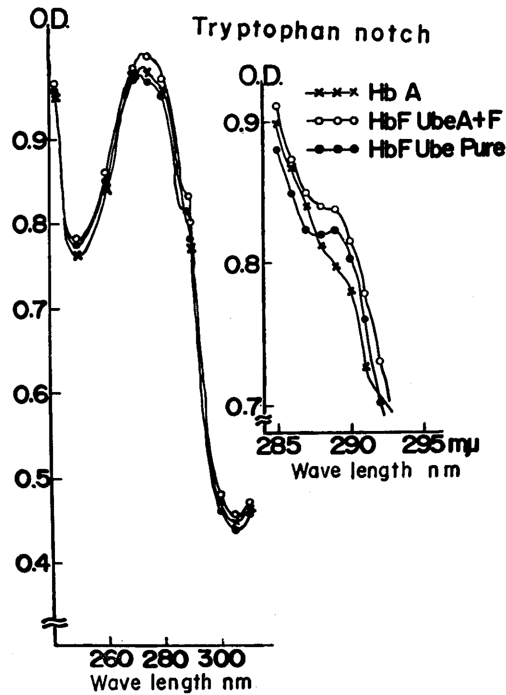

Fig. 3. Ultraviolet spectroscopy of baby's hemolysate (Hb F Ube A+F) and purified fraction of the abnormal hemoglobin (Hb F Ube pure) in comparison with that of the hemolysate of a normal adult subject (Hb A).

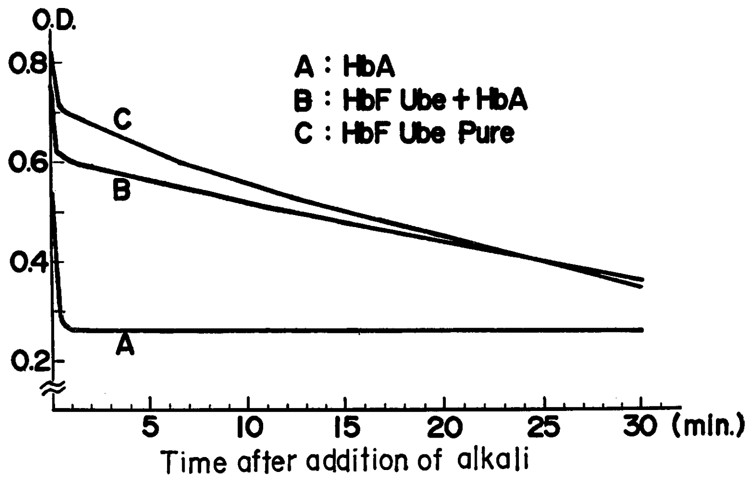

Fig 4. Alkali denaturation test (Jonxis).
A: Hemolysate of a normal adult subject (Hb A).
B: Hemolysate of the baby (Hb F Ube+Hb A).
C: Purified fraction of the abnormal hemoglobin of the baby (Hb F Ube pure).

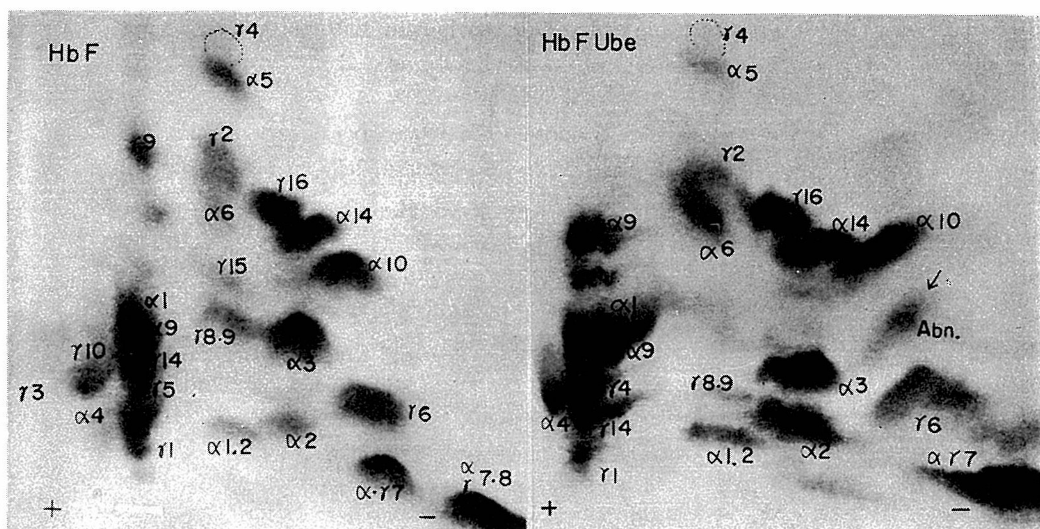

Fig. 5. Fingerprint of Hb F Ube (right) in comparison with that of normal Hb F (left).
Arrow indicates the abnormal peptide spot.

DISCUSSION

It is apparent from the fore-going account that this abnormal hemoglobin belong to the group of fetal hemoglobins which are characterized by alkali-resistance and a salient tryptophan notch. Progressive decrease of the content of the abnormal hemoglobin in the hemolysates with the advance of post-natal growth, and its complete disappearance on approximately the 100 th neonatal day are also notworthy. These properties and attitudes of the abnormal hemoglobin evidence its γ chain anomaly. Accordingly, the abnormal hemoglobin is warranted the name Hb F Ube.

The abnormal fetal hemoglobin is slower than Hb F (normal fetal hemoglobin) in the electrophoretic migration to the anode at pH 7.0 and 8.6. This suggests a one-point mutation, concerning an amino acid substitution in the γ chain gene resulting in diminution of negative charge, such as displacement of a neutral amino acid residue by a basic one or replacement of an acidic amino acid residue by a neutral or basic one.

The abnormal peptide spot visualized on the fingerprint map of this hemoglobin was composed of Leu, Gly and Lys. Probably, substitution of Lys for a neutral or acidic amino acid residue has occurred in the abnormal γ chain, because this would cause a diminution of negative charge which is consistent with the electrophoretic properties of this abnormal hemoglobin. Scrutiny of the amino acid

sequence of the γ chain[17] with special reference to the sites where Leu and Gly lie adjacent discloses the following 3 loci.

(1) ......$\overset{26}{\mathrm{Glu}} \cdot \overset{27}{\mathrm{Thr}} \cdot \overset{28}{\mathrm{Leu}} \cdot \overset{29}{\mathrm{Gly}} \cdot \overset{30}{\mathrm{Arg}}$......

(2) ......$\overset{69}{\mathrm{Gly}} \cdot \overset{70}{\mathrm{Ser}} \cdot \overset{71}{\mathrm{Leu}} \cdot \overset{72}{\mathrm{Gly}} \cdot \overset{73}{\mathrm{Asn}}$......

(3) ......$\overset{104}{\mathrm{Arg}} \cdot \overset{105}{\mathrm{Leu}} \cdot \overset{106}{\mathrm{Leu}} \cdot \overset{107}{\mathrm{Gly}} \cdot \overset{108}{\mathrm{Asn}}$......

No sites in γ chain other than loci (1)～(3) can produce the relevant abnormal peptide spot (composed of Leu, Gly and Lys) on the fingerprint map by one-point mutation.

According to the triplet code theory,[18] Thr (RNA code: ACA, AGG) may be displaced by Lys (RNA code: AAA, AAG) due to an error in taking the bases (C→A). If this occurs at locus (1) an amino acid sequence: ...$\overset{26}{\mathrm{Glu}} \cdot \overset{27}{\mathrm{Lys}} \cdot \overset{28}{\mathrm{Leu}} \cdot \overset{29}{\mathrm{Gly}} \cdot \overset{30}{\mathrm{Arg}}$..., which would produce an abnormal peptide $\overset{28}{\mathrm{Leu}} \cdot \overset{29}{\mathrm{Gly}} \cdot \overset{30}{\mathrm{Arg}}$ by tryptic digestion will result. However, this abnormal peptide differs from the one actually obtained by having Arg at site 30 rather than Lys. Lys can not substitute for Arg. Therefore, locus (1) should be abandoned as a possible site of one-point mutation for the abnormal γ chain of Hb F Ube.

At locus (2), residue Ser (RNA code: UCA, UCC, AGU, AGC) may be replaced by Phe (RNA code: UUU, UUC) or Arg (RNA code: AGA, AGG). Substitution of Phe for Ser does not cause the diminution of negative electric charge, but displacement of Ser by Arg will result in the decrease of negative charge. However, the peptide (Leu, Gly and Lys) cannot be released by trypsin digestion ($\overset{71}{\mathrm{Leu}} \cdot \overset{72}{\mathrm{Gly}} \cdot \overset{73}{\mathrm{Asp}} \cdot$ ... will come forth instead). $\overset{73}{\mathrm{Asp}}$ is not replaced by Lys. As a corollary, the mutation at locus (2) is inconsistent with Hb F Ube.

However, at locus (3), the Asn (RNA code: AAU, AAC) residue can be displaced by Lys (RNA code: AAA, AAG), and this will result in diminution of the negative electric charge in the relevant γ chain. Because trypsin splits a polypeptide in such a way that carboxylic acid redicals may be exposed at residues Arg and Lys, this substitution of Lys for Asn will produce a peptide $\overset{105}{\mathrm{Leu}} \cdot \overset{106}{\mathrm{Leu}} \cdot \overset{107}{\mathrm{Gly}} \cdot \overset{108}{\mathrm{Lys}}$ from the abnormal γ chain by trypsin digestion. This peptide is composed of three amino acids, namely Leu, Gly and Lys, all quite similar in composition to the abnormal peptide actually observed on the fingerprint map. Unfortunately, the peptides $\overset{109}{\mathrm{Val}} \cdot \overset{110}{\mathrm{Leu}} \cdot \overset{111}{\mathrm{Val}}$ ...$\overset{116}{\mathrm{Ileu}} \cdot \overset{117}{\mathrm{His}} \cdot \overset{118}{\mathrm{Phe}} \cdot \overset{119}{\mathrm{Gly}} \cdot \overset{120}{\mathrm{Lys}}$ which one would expect to be released from the abnormal γ chain (γ Tp-12: $\overset{105}{\mathrm{Leu}} \cdot \overset{106}{\mathrm{Leu}} \cdot \overset{107}{\mathrm{Gly}} \cdot \overset{108}{\mathrm{Asn}} \rightarrow \mathrm{Lys} + \overset{109}{\mathrm{Val}} \cdot \overset{110}{\mathrm{Leu}} \cdot \overset{111}{\mathrm{Val}} \cdot \overset{112}{\mathrm{Thr}} \cdot \overset{113}{\mathrm{Val}} \cdot \overset{114}{\mathrm{Leu}} \cdot \overset{115}{\mathrm{Ala}} \cdot \overset{116}{\mathrm{His}} \cdot \overset{117}{\mathrm{His}} \cdot \overset{118}{\mathrm{Phe}} \cdot \overset{119}{\mathrm{Gly}} \cdot \overset{120}{\mathrm{Lys}}$) could not be located on the fingerprint map. Perhaps, these might have been lost together in the core. Amino acid analysis of the abnormal peptide is not exactly consistent

with Leu • Leu • Gly • Lys, but lacks one Leu residue.

It is presumed that Hb F Ube is most likely to be an abnormal fetal hemoglobin which has a substitution of Lys for Asn (γ 108) in its γ chain. The properties of this abnormal hemoglobin are adequately accounted for by this amino acid substitution.

Heretofore, several abnormal hemoglobins with a γ chain anomaly have been recorded in the United States and Europe. They are Hb Alexandra (γ 12 Thr→Lys)[19)20)], Hb Texas I (γ 5 Glu→Lys)[21)] Hb Texas II (γ 6 Glu→Lys)[22)], Hb F Malta (γ 17 His→Arg)[23)] and Hb Hull (γ 121 Glu→Lys)[24)]. It is interesting that they are all slow moving. Hb F Ube (γ 108 Asn→Lys ?) should be classified in the same category. Amino acid substitution has not yet been suggested for Hb F Akashi and F Fukuoka. It may be worth-while to mention that the Hb A_2 content of the hemolysate of the baby with Hb F Ube was relatively high.

CONCLUSION

During the course of electrophoretic screening of the hemolysates prepared from cord blood samples collected in Ube, an alkali-resistant slow-moving hemoglobin was discovered. It had a salient tryptophan notch (289 nm) in the ultraviolet region. The fingerprint map of the tryptic digest of the globin of this abnormal hemoglobin is characterized by an abnormal peptide spot (composed of Leu, Gly and Lys) which was visually distinctive in the cathodal area between the spot (α Tp-10) and the spot (γ Tp-6). From the results of the amino acid analysis of this abnormal peptide, it was concluded that this hemoglobin (Hb F Ube) had a substitution of Lys for Asn (γ 108) in its γ chain.

REFERENCES

1) Körber, E.: Inaugural dissertation, Dorpat (1866), cited by Bischoff, H.: Resistance of human hemoglobin with special regard to infant. *Ztschr. Exper. Med.*, **48**: 427-489, 1926.
2) White, J.C. and Beaven. G.H.: Foetal hemoglobin. *Brit. Med. Bull.*, **15**: 33-39, 1959.
3) Lehmann, H. and Huntsman, R.G.: *Man' s Haemoglobins.* North Holland (Amsterdam), 1966.
4) Schroeder, W.A., Huisman, T.H.J., Shelton, J.R., Shelton, J.B., Kleihauer, E.F., Dozy, A.M. and Robberson, B.: Evidence for multiple structural genes for the γ chain of human fetal hemoglobin. *Proc. Nat. Acad. Sci, (USA)*, **60**: 537-544, 1968.
5) Ingram, V.M.: Abnormal human hemoglobin III. The chemical difference between normal and sickle cell haemoglobins. *Biochim, Biophys. Acta.* **36**: 402-411, 1959; Ingram, V.M.: *Hemoglobin and its abnormalities.* Thomas (Springfield). 1961.
6) Gillespie, J.E.O.N., White, J.C., Beaven, G.H., Gratzer, W.B., Shooter, E.M., Parkhouse, R.M. A haemoglobin with unusual alkaline-denaturation properties in a Turkish-Cypriot woman. *Nature*, **184**: 1876-1877, 1959.

7) Shibata, S. and Ueda, S.: List of abnormal hemoglobin recorded in the world. *Bull. Yamaguchi Med. School*, **17**: 1-22, 1970.
8) Ishiwara, Y.: γ-chain variant of abnormal hemoglobin discovered from a resident in Akashi (Hb F Akashi). *Acta Haem, Jap.*, **30**: 640-640, 1967.
9) *Personal communication* from Ohta, Y.
10) Jonxis, J.H.P. and Huisman, T.H.J.: *A Laboratory Manual on Abnomal Haemoglobins.* Blackwell Scientific Pub. (Oxford, Edinburgh), 1968.
11) Shibata, S. and Iuchi, I.: A simple technique of agar gel electrophoresis for rapid separation of hemoglobins. *Acta Haem: Jap.*, **24**: 51-58, 1961.
12) Brinkman, R. and Jonxis, J.H.P.: The occurrence of several kinds of hemoglobin in human blood. *J. Physiol.*, **85**: 117-127, 1935.
13) Anson, M.L. and Mirsky, A.E.: protein coagulation and its reversal. The preparation of insoluble globin, soluble globin and heme. *J. Gen. Physiol.*, **13**: 469-476, 1930.
14) Baglioni, C.: An improved method for the fingerprinting of human hemoglobin. *Biochim. Biophys. Acta*, **48**: 392-396, 1961.
15) Spackman, D.H.: *Amino acid analysis and related procedures.* (New York, London), 1967, p. 3-15.
16) Takahashi, K.: Enzymatic decomposition of protein and polypeptides. *Protein-Nucleic acid-Enzyme* (Tanpakushitsu-Kakusan-Koso), **8**: 91-95, 1963.
17) Huisman, T.H.J. and Schroeder, W.A.: *New Aspects of the Structure, Function and Synthesis of Hemoglobins.* Butterworth (London), 1971.
18) Harris, H.: *The principles of Human Biochemical Genetics.* North-Holland (Amsterdam, London), 1970.
19) Vella, F.. Ager, J.A.M. and Lehmann. H.: New variant of human foetal hemoglobin. *Nature*, **183**: 31-32, 1959.
20) Lehmann, H., Carrell, R.W.: Variations in the structure of human hemoglobin with particular reference to the unstable hemoglobins. *Brit. Med. Bull.*, **25**: 14-23, 1969.
21) Jenkins, G.C., Beale, D., Black, A.J., Huntsman, R.G. and Lehmann, H.: Hemoglobin F. Texas I ($\alpha_2\gamma_2{}^{5\,Glu\rightarrow Lys}$): a variant of hemoglobin F. *Brit. J. Haem*at. **13**: 252-255, 1967.
22) Larkin, I.L.M., Braker, T., Lorkin, P.A., Lehmann, H., Black, A.J. and Huntsman, R.G.: Hemoglobin F Texas II ($\alpha_2\gamma_2{}^{6\,Glu\rightarrow Lys}$) the second of the hemoglobin F Texas variants. *Brit. J. Haemat.*, **14**: 233-238, 1968.
23) Cauchi, M.N., Clegg, J.B. and Weatherall, D.J.: Haemoglobin F (Malta): a new foetal haemoglobin variant with a high incidence in Maltose infants. *Nature*, **223**: 311-313, 1969.
24) Sacker, L.S., Beale, D., Black, A.J., Huntsman; R.G., Lehmann, H., and Lorkin, P.A.: Hemoglobin F Hull (Gamma-121 glutamic acid→Lysine), homologous with hemoglobins O Arab and O Indonesia. *Brit. Med. J.*, **3**: 531-533, 1967.