Hemoglobin M's of the Japanese

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Seven years ago there was a consensus of opinions of hematologists that hemoglobinopathy did not exist in Japan. However, the surveys on abnormal hemoglobins carried out for the recent ten years[1][2] have overthrown this conception. Indeed hemoglobinopathy is rare in Japan, but there are a great variety of abnormal hemoglobins distributed among the Japanese. No less than 28 kinds of abnormal hemoglobins have hitherto been recorded in this country.[3]

Hemoglobin M is the most note-worthy of these abnormal hemoglobins, because it is associated with overt clinical symptoms. For hundreds of years a strange disease called "Kuchikuro" (black mouth) has been endemic in a restricted area of Northern Japan.[4][5] Its etiology was finally established in 1960 when Hemoglobin M_{Iwate} was discovered from the blood of a patient with this disease.[6] Later other hemoglobin M's were found in various parts of this country. These hemoglobins are providing the research workers who are interested in the physiology of hemoglobin with valuable materials.

HISTORICAL

About 160 years ago a cyanotic baby ("Kuroko" : black child) was born to a couple who lived in a northwestern area of Iwate prefecture (Fig. 1). Such cyanotic subjests have grown in number from generation to generation until 1950's when it was estimated that there were approximately 70 patients residing scatteredly in several districts of Iwate prefecture (Shiwa, Ikkatai and Mido). One of the patients attracted the attention of Sasaki, who in collaboration with Kimura[7] published the first case report of this abnormality in 1936. Shikinami

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Dedicated to Emeritus Prof. Tanemoto Furuhata for the celebration of the 77th anniversary of his birth.
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and Hosokawa (1937)[8] reported the second case, but confused it with "hereditary Addison's disease." Tanobe[9] who made fundoscopic examination of the eyes of the patients suggested a dominant inheritance. Since 1950 an extensive study covering all the branches of medical sciences (symptomatology, hematology, blood chemistry, blood groups, spectroscopy of hemoglobin, genetic survey, etc.) had been carried out by Tamura and his associates[4][10][11] of the Iwate Medical College. They noticed that patient's blood was as black as Japanese soy sauce and could not be turned red as the venous blood of a normal subject even by vigorous aeration. Furthermore, they discovered an important abnormality of the spectroscopic character of patient's hemoglobin : To their surprise the acid methemoglobin type hemolysate of the patient showed spectroscopically neither the peak at 630 mμ nor the depression around 600 mμ, each of which was characteristic of the acid methemoglobin of normal subject. They[12] separated heme and globin from the hemoglobin solution of the patient by Anson-Mirsky's technique in order to subject the heme solution to column chromatography and the globin to amino acid analysis. They alleged that the heme was abnormal in the patient's hemoglobin, because they noticed two layers of pigments, the greenish and the brownish, when they put the patient's heme solution (in acetone) on the column of calcium carbonate. These layers of unusual color were not visible with the heme solution of normal hemoglobin. Acid hydrolysate of patient's globin failed to give any abnormality by two dimensional paper chromatographic analysis. Accordingly, Tamura[10][11] came to the conclusion that the etiology of this desease was related to production of a hemoglobin possessing abnormal porphyrin nuclei extremely prone to spontaneous oxidation yielding hematin-like complex compounds. Thus, he named the malady "black blood disease" or "hereditary nigremia."

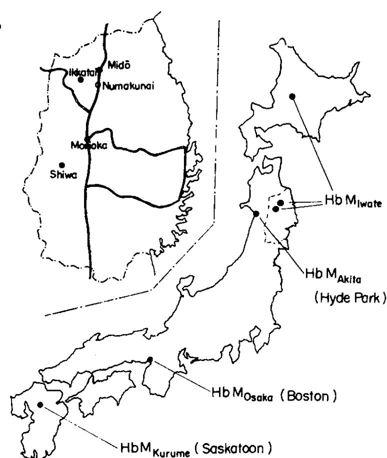

Fig. 1. Geographical distribution of Hb M diseases in Japan. Black spots indicate the towns where the patients were detected. At the upper left corner an enlarged map of Iwate prefecture is presented.

At about the same period Hörlein and Weber (1948)[13] discovered in Elberfeld, Germany, a family of congenital cyanosis unrelated to hereditary methemoglobinemia which had been well known till then. They examined the patient's acid methemoglobin type hemolysate spectroscopically, prepared globin from it, and successfully reconstituted the original methemoglobin possessing a peculiar absorption curve (lacking both of the peak at 630 mμ and the depression around

600 mμ by mixing the patient's globin with hemin of a normal person. On the basis of this experiment they concluded that the patients were producing a modified methemoglobin (eine neue Modifikation des Methämoglobins) which was entirely different from the normal human methemoglobin. This methemoglobin was classified into abnormal hemoglobins under the name of "hemoglobin M" by Singer (1955),[4] who attached special importance to the abnormality of its globin. M (Hb M) had not been obtained in pure form until 1958, when Gerald[15] successfully isolated Hb M_{Boston} (a variant of Hb M's) by starch block electrophoresis of patient's methemoglobin type hemolysate. His experiment elucidated for the first time the presence of an abnormal hemoglobin (Hb M) which was chocolate brown in color along with the normal adult hemoglobin (Hb A) which was red in patient's hemolysate. Tamura[6] was acquainted with Hörlein-Weber's report, but he was not influenced, being still confident of his conception that hereditary nigremia in Iwate prefecture was a disease caused by the production of a hemoglobin possessing abnormal heme.

It was in 1960 that hereditary nigremia was established as one of the variants of Hb M disease. Early in this year Shibata and his associates[6] who were engaged in hemoglobinopathy survey in Ube received a blood sample of hereditary nigremia from Tamura, and demonstrated a stripe of unusual color beside a red stripe of normal adult hemoglobin (Hb A) by agar gel electrophoresis of the hemolysate prepared from the patient's blood. The abnormal stripe was chocolate brown, and migrated to the anode side of Hb A at pH 8.6 as well as at pH 7.0. Further study of this abnormal stripe by spectroscopy, chromatography and starch block electrophoresis carried out in Ube revealed the presence of a new type of Hb M in patient's hemolysate. This was the hemoglobin which is now widely known as Hb M_{Iwate}. Hemoglobin M_{Iwate} attracted the attention of a number of hematologists and biochemists in the foreign countries as well as in Japan, i. e. its chemistry was studied by Gerald,[17] Lehmann,[18][19] Konigsberg,[19] Motokawa,[20] Kikuchi[21] and Shimizu.[22] However, Shibata, Miyaji, Iuchi and Tamura (1963, 1964)[23][24][25][26] are credited with the first successful establishment of the amino acid substitution in this hemoglobin.

The year 1960 is of special commemoration in the history of hemoglobin survey in Japan, because another type of Hb M disease beside hereditary nigremia was discovered in this country. This year, in Kurume, a city in Kyushu situating far away west-wards from Iwate prefecture (Fig 1), a cyanotic boy was seen by Kimura and his colleagues.[27] They performed spectroscopic study of the acid methemoglobin type hemolysate of the patient and diagnosed that the boy had Hb M disease. Yamaoka and others[28] isolated his Hb M by starch block electrophoresis. Shibata, Miyaji and Iuchi[29] called the hemoglobin Hb M_{Kurume}, and elucidated its amino acid substitution in 1962.

Fig. 2. Agar gel electrophoresis (pH 7.0) of the hemolysate of Hb M_{Iwate} disease (hereditary nigremia).[33][34] Note the chocolate brown stripe of Hb M_{Iwate} (anode side) in comparison with the red color of Hb A strip (the cathode side).

Fig. 4. Amberlite IRC 50 chromatography (Huisman) of the hemolysate of Hb M_{Iwate} disease.[34] Note the chocolate brown color of the layer of Hb M_{Iwate} separated at the top of the column. The Hb A layer which is seen at the bottom is red.

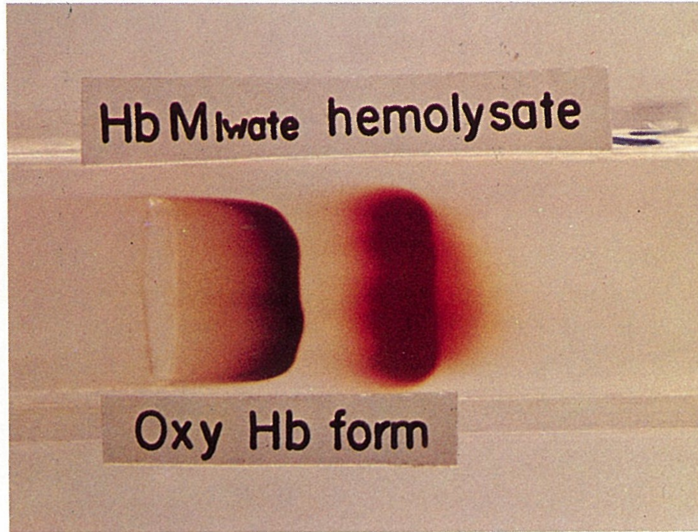

Fig. 2.

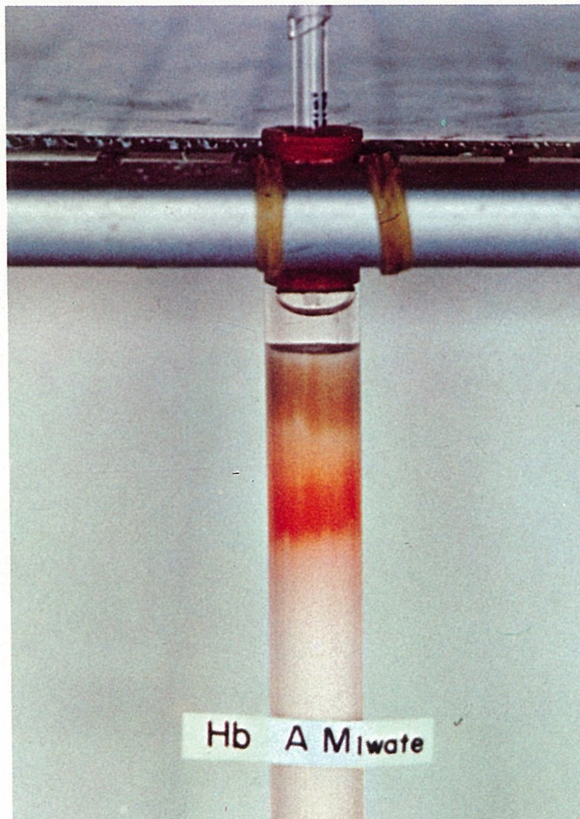

Fig. 4.

Next year (in 1963) the third sort of Hb M was encountered in Osaka. Hayashi and others[30] detected a chocolate brown hemoglobin in a hemolysate of a cyanotic man by agar gel electrophoresis. The hemoglobin was designated Hb M_{Osaka}. This was identified as a variant of Hb M's by chemical study (amino acid substitution) three years after its discovery by Shimizu and his associates.[31]

Quite recently (in 1966) the fourth variant of Hb M was discovered in Akita prefecture by Shibata, Miyaji, Karita and others.[32] They named the hemoglobin Hb M_{Akita}. Akita prefecture is adjacently situated to Iwate prefecture. They accomplished its chemical study in 1967. This hemoglobin was different from Hb M_{Iwate}, being a new variant which had never been recorded before.

As understood from the fore-going account of historical review there are at present four kinds of Hb M's in Japan: Hb M_{Iwate}, Hb M_{Kurume}, Hb M_{Osaka} and Hb M_{Akita}. It is the purpose of this paper to describe the characteristic properties of these Hb M's and symptoms associated with these abnormal hemoglobins, together with a discussion of their comparison with the Hb M diseases distributed throughout the world.

ELECTROPHORESIS

Agar gel electrophoresis (pH 7.0)[33] is the most convenient and simplest method for demonstrating the Hb M's of Japanese. Hb M is separated from Hb A as a chocolate brown stripe migrating to the anode side of red Hb A stripe[34][35] (Fig 2). However, there is one point to be made special mention of; Hb M_{Kurume} and Hb M_{Akita}, which are of β chain anomaly, are not always separable from Hb A by direct application of O_2Hb type hemolysate to the agar, unless fresh blood immediately after collection is used for the preparation of hemolysate. Hb M_{Iwate} and Hb M_{Osaka}, which have α chain anomaly, are easily demonstrable by agar gel electrophoresis (pH 7.0 and 8.6) with O_2Hb type hemolysate. On the contrary, Hb M_{Kurume} and Hb M_{Akita} produce a stripe sharply delineated from Hb A stripe, if methemoglobin type hemolysate (made by addition of appropriate amount of ferricyanide to O_2Hb type hemolysate)[34] is subjected to agar gel electrophoresis. Starch block electrophoresis (pH 7.0)[34] of the methemoglobin type hemolysate affords the best way for the purification of Hb M's of Japanese (Fig. 3). Moving-boundary electrophoresis and paper electrophoresis are not useful, for separation of Hb M is seldom successful by these procedures.*

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* Exception: Obara and his associates[36] were successful in separating Hb M_{Iwate} by paper electrophoresis (pH 8.9), and Hansen and his coworkers[37] noticed the peak of Hb $M_{Gothenburg}$ (identical with Hb M_{Osaka}) on Tiselius electrophoresis (pH 6. 8).

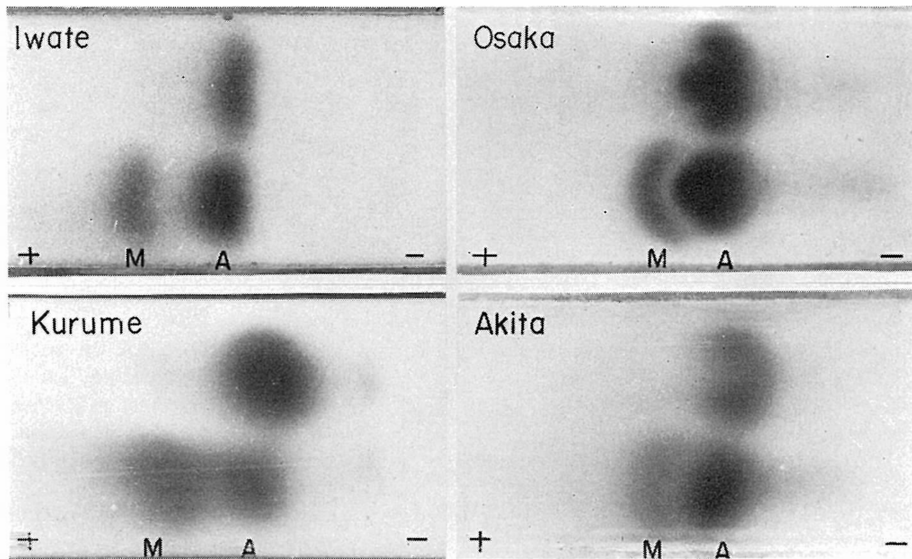

Fig. 3. Starch block electrophoresis of Hb M's of Japanese.[32)34)44)]
On each starch block the top row and the bottom row refer to the metHb type hemolysate of a normal subject and of a patient with Hb M disease, respectively.
A : metHb A. M : metHb M
MetHb M_{kurume} was greenish in color, while metHb M_{Iwate} and metHb M_{Osaka} were grey. MetHb M_{Akita} was grey brown.

CHROMATOGRAPHY

Chromatography on the column of Amberlite IRC 50 (or CG50, XE64) of O_2Hb type hemolysate by eluation with ammonium phosphate buffer solution (pH 7.7) is the most favored.[20)22)30)] Amberlite IRC 50 chromatography for separation of Hb F which is recommended by Huisman[38)] is also useful. By the latter method Hb M forms a dark gray layer above the red layer of Hb A.[6)34)] Usually two layers are seen, namely Hb M (dark gray, at the top) and Hb A (red, at the bottom) (Fig. 4), but additional several layers of varied colors may occasionally appear above and below these when hemolysate prepared from aged blood sample is applied to the column. However, Hb M_{Akita} is an exception. The fresh hemolysate (O_2Hb type) of Hb M_{Akita} disease always yields three layers: (1) Hb M_{Akita} (top), (2) Hb A (middle) and (3) minor hemoglobin component which is red (bottom).[32)] (Fig. 5).

From carboxymethylcellulose column of Huisman Hb M_{Iwate} was eluted between Hb A_0 and Hb A_2, but its isolation was not satisfactory.[39)]

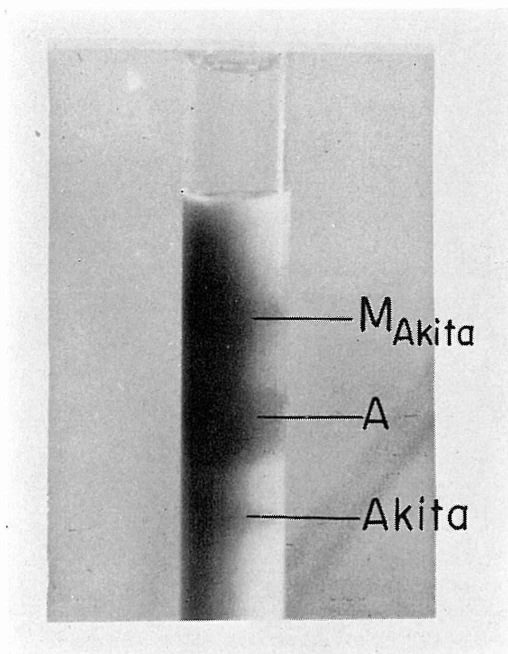

Fig. 5. Amberlite IRC 50 chromatography of the hemolysate of Hb M_{Akita} disease. The Hb M_{Akita} layer, which is seen at the top of the column, is grayish-brown in color, while the lower layers referring to Hb A and Hb Akita (the minor component of hemoglobin contained in the hemolysate) are red.
By electrophoresis, Hb Akita (red in color) migrates to the anode between Hb A and Hb A_2 in agar gel (pH 8.6).

SPECTROSCOPY

Most of the abnormal hemoglobins are red when oxygenated, and it is almost impossible to discriminate them from the normal adult hemoglobin (Hb A) by spectroscopic examination. Hb M forms an exception to this general rule. The blood and hemolysate containing Hb M are "black", assuming an unusual color easily recognizable with the naked eye. Spectroscopy of the hemolysate, either oxygenated or oxidized, discloses distinct abnormality of the absorption curve, and solutions of purified Hb M and its derivatives also show characteristic patterns of light absorption which are significantly different from those of the relevant derivatives of Hb A. Spectroscopy is therefore a procedure of paramount importance for the characterization of Hb M's. Thus the Hb M's of Japanese were earnestly studied spectroscopically by the groups of Tamura,[10)11)] Shibata,[6)40)41)42)] Motokawa[20)] and Hayashi.[30)]

(1) Spectrpscopy of the hemolysate.[20)34)] As pointed out early by Tamura and his associates,[10)11)] in the oxygenated form (O_2Hb type) of the hemolysate of the patient with Hb M disease the α (575 mμ) and β (540 mμ) peaks of the absorption curve are more or less indistinct, because the depression between the peaks is shallower and their feet are higher than in the oxygenated normal hemolysate (Fig. 6). The difference becomes more salient when oxidized forms

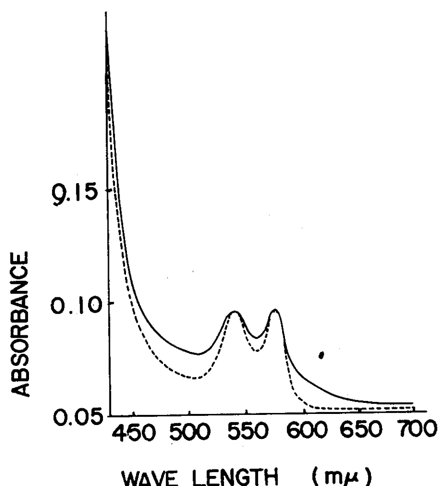

Fig. 6. Absorption curve of oxyhemoglobin type hemolysate of Hb M disease (Hb M_{Iwate}) disease.
Solid line: hemolysate (pH 8.6) of a patient with Hb M_{Iwate} disease. Dotted line : hemolysate (pH 8.6) of a normal subject.

(methemoglobin type) of hemolysates are compared. Oxidized normal hemolysate has a distinct peak at 630 mμ and remarkable depression around 600 mμ. By contrast, the oxidized hemolysates of the Hb M diseases of the Japanese are unanimously characterized by the lack of this kind of peak and depression, showing an inflection at 630 mμ which passes into a somewhat horizontal line reaching a peak around 500 mμ (Fig. 7).[20][27][30][32][34]

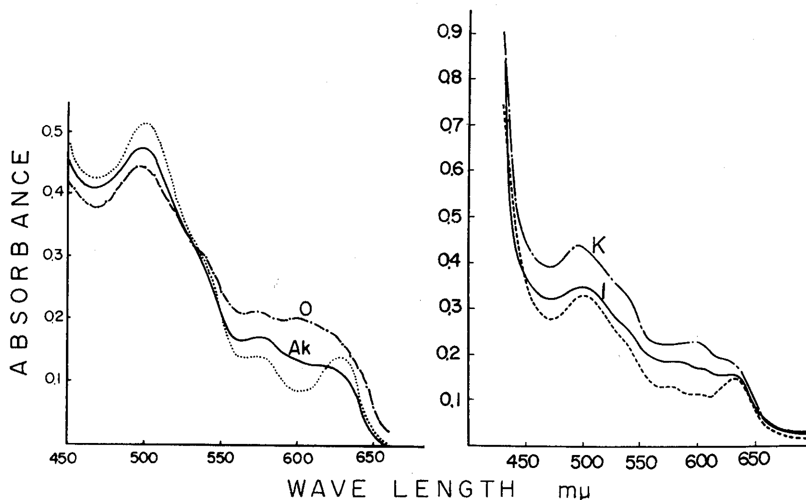

Fig. 7. Methemoglobin type hemolysates (pH 6.5–7.0) of Hb M diseases. AK: Hb M_{Akita} disease. I: Hb M_{Iwate} disease. K: Hb M_{Kurume} disease. O: Hb M_{Osaka} disease Dotted or broken line without notation: MetHb A.

(2) Oxygenated Hb M's. Oxygenated hemoglobins (O_2Hb) of Hb M_{Iwate} and Hb M_{Osaka} (of α chain anomaly) can be purified by agar gel electrophoresis of the O_2Hb type hemolysate of the patients.[30][34] They are also separable from

O_2Hb A by column chromatography (Amberlite XE 64 or CG 50).[20][22][31] The samples of O_2Hb M's purified by these procedures are the material suitable for spectroscopy. However, contamination with a certain amount of metHb M's is inevitable because O_2Hb M's are partly auto-oxidized during the process of purification. Accordingly, check should always be made for the contamination by adding a minute amount of saturated potassium cyanide solution to the purified sample of O_2Hb M's. Hb M_{Kurume} and Hb M_{Akita} (of β chain anomaly) are more vulnerable than Hb M_{Iwate} and Hb M_{Osaka}. They are prone to deterioration during electrophoresis.[32] So, it was almost impossible for us to trace the absorption curves of their oxygenated forms (O_2Hb M's) by direct application of spectroscopy to the purified O_2Hb M sample. In Hb M_{Akita}, patient's hemolysates of O_2Hb type and metHb type were subjected to spectroscopy, and their content of hemoglobin (Hb A plus Hb M_{Akita}) was measured by alkali hemochrome method.[42][43] The Hb M_{Akita} contents of the patient's hemolysate were calculated by the comparison of observed optical density (at 600 mμ) of patient's metHb type hemolysate with the optical densities (at 600 mμ) of the solutions of metHb M_{Akita} and metHb A, which were the same in concentration as the patient's hemolysate. Then the absorptions caused by O_2Hb A corresponding to the calculated content of Hb A in the patient's hemolysate were subtracted from the absorption curve of the patient's O_2Hb type hemolysate over the whole range of visual spectrum (350 to 700 mμ). The resultant curve was thought to be referable to the absorption curve of O_2Hb M_{Akita}.[44]

The absorption curves of O_2Hb M's of the Japanese are presented in Fig. 8.

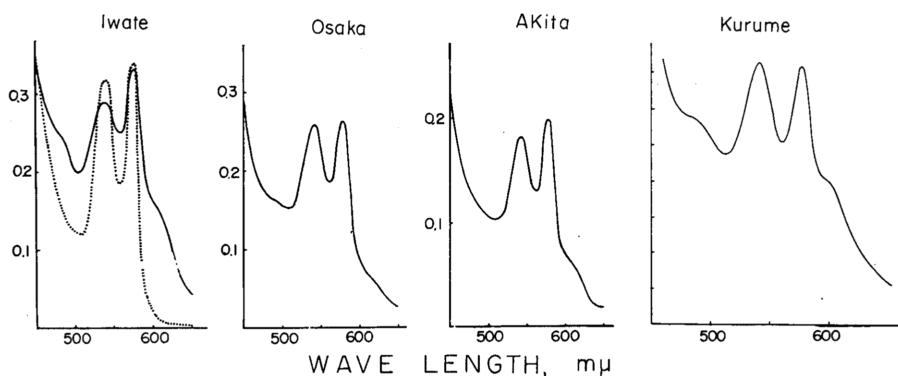

Fig. 8. Absorption curves of oxyhemoglobin M's of Japanese. Dotted line represents the absorption curve of oxyhemoglobin A.

They are all easily differentiated from that of O_2Hb A, because they are characterized by less salient protrusion of the α (576 mμ) and the β (540 mμ) peaks and the presence of an inflection around 600–610 mμ. Their Soret peaks are

seen around 408 mμ with slight dislocation to the side of shorter wave length than in O_2Hb A (415 mμ). This peculiar shape of the absorption curves exhibiting larger light absorption in the range outside of the α and β peaks confers on these abnormal hemoglobins a darker hue, which results in characteritically chocolate brown color.

Nevertheless, there is really a delicate difference in color from variant to variant, e. g., O_2Hb M_{Akita} looks lighter than O_2Hb M_{Iwate}. This will be perceived from Fig. 9, in which typical color curves (Heilmeyer)[95] of O_2Hb M_{Iwate}, O_2Hb M_{Osaka} and O_2Hb M_{Akita} are depicted.

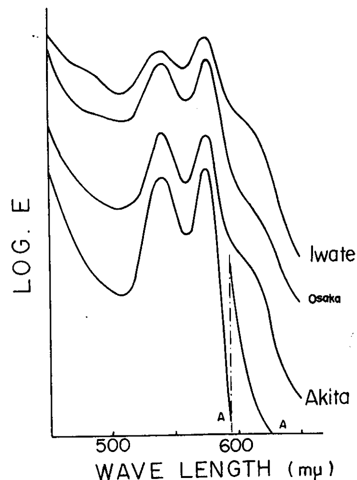

Fig. 9. Typical color curves of oxyhemoglobin M's of Japanese. E: absorbance. A: Oxyhemoglobin A.

(3) Deoxygenated Hb M's. O_2Hb M's are reduced to deoxygenated forms by addition of sodium dithionite.[40] Both of the deoxygenated forms of Hb M_{Iwate} and Hb M_{Osaka} (which are of α chain anomaly) show an absorption peak possessing its maximum at 560 mμ.[20][40] However, they are discriminated from that of deoxygenated Hb A by their flatter shape and by the presence of a slightest inflection around 600 mμ[40] (Fig. 10).

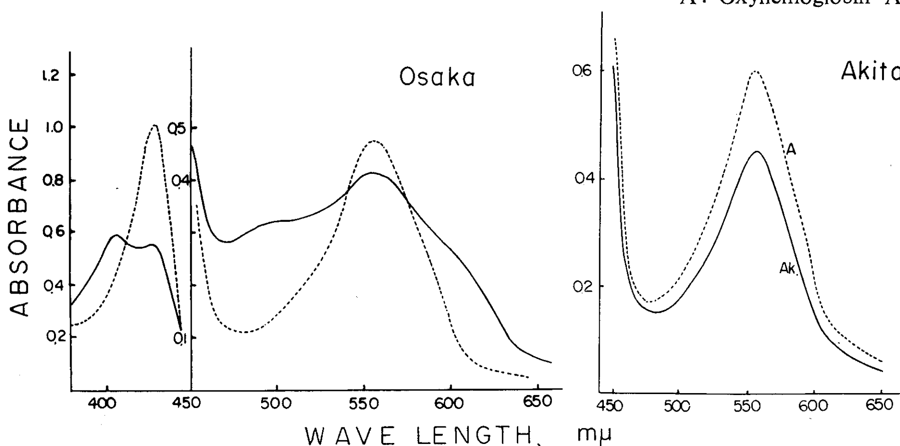

Fig. 10. Absorption curves of deoxygenated hemoglobin M's (Hb M_{Akita} and Hb M_{Osaka}). Note that there is a considerable difference in the shape of curves between Hb M_{Osaka} and Hb A.
A (or broken line): Deoxygenated Hb A. AK (or solid line): Deoxygenated Hb M_{AKita} or Hb M_{Osaka}.

In addition the Soret peak is abnormal, being split into two maximums, at 429 mμ (conforming with the Soret peak of deoxygenated Hb A) and at 406 mμ (reminiscent of the Soret peak of methemoglobin A or methemoglobin M).[20][96]

This is interpreted that these Hb M's are not completely reduced by dithionite. According to Motokawa and his colleagues[20] half a molecule of Hb M_{Iwate} was instantly reduced by dithionite, but the remaining moiety was resistant, and it took about 50 hours at room temperature to reach complete reduction of a whole molecule. It deserves special mention that the absorption spectrum of the final product was indistinguishable from that of deoxygenated Hb A.[20]

Hb M_{Kurume} and Hb M_{Akita} (which are of β chain anomaly) react with dithionite rapidly and completely.[32][34] It is very hard to distinguish deoxygenated forms of these Hb M's from deoxygenated Hb A (peak: 560 mμ, and Soret peak: 430 mμ) spectroscopically.

Table I. The absorption peaks of the derivatives of Hb M's of the Japanese

Oxy-Hb Type pH 7.0~7.2	Hb M_{Iwate}	610 (Inf; 6.7), 576 (14.5), 540 (13.1), 408 (101.4)
	Hb M_{Osaka}	604 (Inf), 577, 540 490, 408
	Hb M_{Akita}	612 (Inf; 5.3), 576 (15.2), 540 (13.9), 408
	Hb M_{Kurume}	600 (Inf.), 576, 539, 484
	Hb A	No inf., 610 (0.5~0.9), 576 (14.7), 540 (14.0), 415 (126.2)
Met-Hb type pH 7.0~7.2	Hb M_{Iwate}	590 (10.5), 490 (14.0), 405 (127.4)
	Hb M_{Osaka}	598 (5.2), 495 (8.42), 405 (115)
	Hb M_{Akita}	610 (Sh; 4.92), 578 (6.42), 496 (10.1), 405
	Hb M_{Kurume}	600, 540 (Sh.), 488, 403
	Hb A	630 (3.4~4.0), 498 (8.4~9.3), 405 (144~157)
Cyan-metHb pH 7.0~7.2	Hb M_{Iwate}	540 (13.5), 415 (88.6)
	Hb M_{Osaka}	535 (12.5), 416 (84.0)
	Hb M_{Akita}	540 (11.2), 414 (97.0)
	Hb A	540 (10.8~11.7), 420 (1C9~112.8)
Reduced Hb pH 6.5~7.0	Hb M_{Iwate}	555 (10.0), 600 (Inf; 7.5), 430 (63), 405 (68.5)
	Hb M_{Osaka}	555 (11.2), 600 (Inf; 7.2), 430 (69), 405 (73.5)
	Hb M_{Akita}	555 (12.6), 430 (Inf; 92.0)
	Hb A	555 (13~13.4), 600, not a protrusion (3.5), 430 (118~135)
Azide metHb	Hb M_{Iwate}	610 (Inf), 573, 540, 403
	Hb M_{Osaka}	610 (Inf; 7.66), 575 (10.3), 533 (12.0), 490 (12.1), 410 (86.6)
	Hb A	610 (Inf; 2.58), 575 (8.84), 542 (11.2), 418 (111.5)

Numerals represent the wave length (mμ) of the absorption maximum of the peak, and numerals in parenthesis (N) refer to millimolar extinction coefficient at the relevant wave length. Notation "Inf." and "Sh" indicate an inflection and shoulder of the absorption curve, respectively. For example, 610 (Inf; 6.7), and 576 (14.5) point to an inflection at 610 mμ with millimolar extinction coefficient of 6.7 and a peak at 576 mμ with millimolar extinction coefficient of 14.5, respectively. The isobestic points between metHb M and metHb A are as follows: --- 627 mμ (Hb M_{Iwate}, pH 7.0), 625 or 632 mμ (Hb M_{Osaka}, pH 7.0), 632 mμ (Hb M_{Akita}, pH 6.5), and 630-633 mμ (? Hb M_{Kurume}, pH 7.0).

The isobestic wave lengths of the absorption curve between O_2Hb A and O_2Hb M (pH 7.0) are as follows: --- 536, 546, 575 and 577 mμ (Hb M_{Iwate}), 530, 554, 560 and 580 mμ (Hb M_{Osaka}), and 527, 560 and 580 mμ (Hb M_{Akita}).

(4) Oxidized Hb M's. Hb M's are converted into relevant oxidized pigments (methemoglobin) by addition of an appropriate amount of ferricyanide to the solutions of oxygenated Hb M's.[40] They are also obtained in pure form by starch block electrophoresis of the patient's methemoglobin type hemolysate.[34]

Their spectroscopy were generally carried out in neutral or acid (pH 7.0~6.5) medium.

The absorption curves of the acid metHb M's are characterized by lack of the peak at 630 mμ and the depression around 600 mμ which are specific for metHb A.

MetHb M's of the Japanese (pH 7,0-6.5) exhibits two protrusions, either distinct or indistinct, around 600 mμ and 480 mμ along with a Soret peak at 405 mμ which is somewhat less soaring than that of metHb A. Protrusion around 600 mμ seems to be more distinctly dislocated to the side of shorter wave length in Hb M_{Iwate} and metHb M_{Akita} than in Hb M_{Kurume} and metHb M_{Osaka}. In reality each of the Hb M's of the Japanese has its own absorption curve, but the difference in shape between some of them (e. g., metHb M_{Iwate} and metHb M_{Akita}) is not so large as it enables us their spectroscopic identification (Fig. 11).

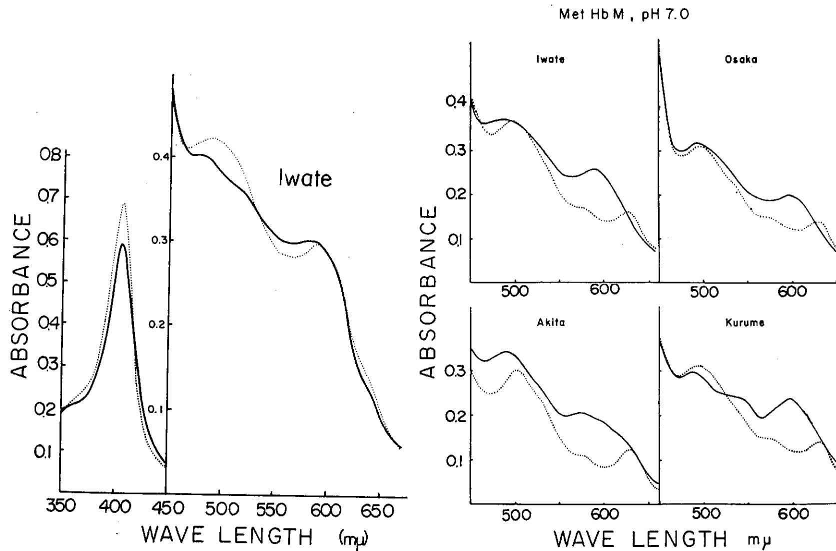

Fig. 11. Absorption curves of methemoglobin M's. Left: absorption curve of metHb M_{Iwate}. Solid line: pH 7.0, Dotted line: pH 6.0. Right: Absorption curves (solid line) of metHb M_{Akita}, metHb M_{Iwate}, metHb M_{Kurume} and metHb M_{Osaka} together with that of metHb A (dotted line).

Gerald and George[45] recommend to test ligand reactivity of heme iron (with cyanide, fluoride, azide, dithionite and hydrogen peroxide) for the characterization of metHb M's. For instance, metHb M_{Iwate} reacted with cyanide slowly, whereas

metHb M_{Kurume} and metHb M_{Akita} underwent rapid reaction.[32][40] However, in our experience usefulness of this test for identification of individual Hb M's is limited, although generally heme iron of metHb M's of β chain anomaly seems to be more ligand reactive than metHb M's of α chain anomaly.

Auto-oxidation (spontaneous methemoglobinization of the normal chain) is more evident in Hb M_{Iwate} and Hb M_{Osaka} than in Hb M_{Akita} and Hb M_{Kurume}.[98]

(5) CyanmetHb M's. Spectroscopically cyanmetHb M_{Iwate} and cyanmetHb M_{Osaka} are different from cyanmetHb A (pH 7.0～6.5).[20][30][104] The absorption peak at 540 mμ were broader and somewhat higher with an inflection around 600 mμ in these cyanmetHb M's. The Soret peak which is to be seen at 420 mμ in cyanmetHb A was shifted to 415 mμ, being more or less obtuse as observed in deoxygenated Hb M's [20][40] (Fig. 12).

According to Hayashi and others [97] the reaction of cyanide with metHb M proceeds by two steps, the first phase concerning the combination of CN^- with the ferric heme iron linked with the normal chain (almost equal in velocity to the reaction of cyanide with metHb A) and the second phase referring to the union of CN^- with ferric heme iron belonging to the abnormal chain. The second step is slow and requires a higher cyanide concentration ($[CN^-]$: 10^{-3}～10^0 for Hb M_{Osaka} and Hb M_{Iwate} and 10^{-5}～10^{-3} for Hb M_{Kurume}, Hb concentration 50 μM as heme.) than that necessary for the CN reaction of ferric heme iron of metHb A ($[CN^-]$: 10^{-6}～10^{-4}) because the affinity to cyanide of the ferric heme iron of the abnormal chain is distinctly small.

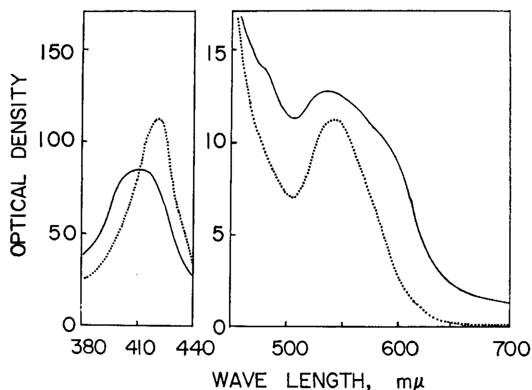

Fig. 12. Absorption curve of cyanmethemoglobin M_{Iwate}. Solid line: cyanmet Hb M_{Iwate}. Dotted line: cyanmet Hb A.

CyanmetHb M_{Akita} closely resembled cyanmetHb A in the shape of absorption curve. The inflection around 600 mμ was indistinct, but the peak at 540 mμ tended to be broader and the Soret peak was slightly dislocated to the shorter wave length (414 mμ).[32] Similar slight abnormality is expected to be seen in cyanmetHb M_{Kurume}.

(6) Azide metHb M's. Azide metHb M_{Iwate} shows an abnormal absorption curve with indistinct α (573 mμ) and β (540 mμ) protrusions, an inflection around 600 mμ and a slightly obtuse Soret peak at 403 mμ (The Soret peak of azide metHb A is at 418 mμ[20][40]) (Fig. 13). Azide metHb M_{Osaka} was slightly different from azide metHb M_{Iwate} in β peak and Soret peak.

(7) Fluoride metHb M's. Motokawa and his associates[20] studied fluoride metHb M_{Iwate}. There was a good agreement of the shape of absorption curves in Soret region (around 404 mμ) between fluoride metHb M_{Iwate} and fluoride metHb A, but in the visible range the light absorption was generally stronger in fluoride metHb M_{Iwate}. The peaks were seen at 600, 485 and 404 mμ (Fig. 14).

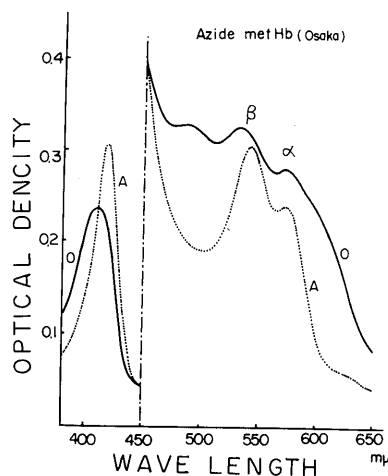

Fig. 13. Absorption curve of azide methemoglobin M_{Osaka}.
Solid line: azide metHb M_{Osaka}.
Dotted line: azide metHb A.

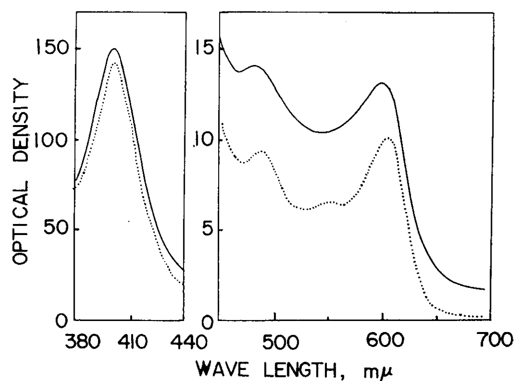

Fig. 14. Absorption curve of fluoride methemoglobin M_{Iwate}. Solid line: fluoride metHb M_{Iwate}.
Dotted line: fluoride metHb A.

(8) Carbonmonoxy-Hb M's (COHb M's). COHb M_{Iwate} exhibits an absorption curve resembling that of O_2Hb M_{Iwate}.[20] It has α (570 mμ) and β (540 mμ) peaks together with an inflection around 600 mμ. Its Soret peak is at 420 mμ with an inflection (400~405 mμ). Similar absorption curve was also seen in COHb M_{Osaka}.[30]

(9) Heme. Shibata and his associates[40] demonstrated that the pyridine hemochromogen prepared from O_2Hb M_{Iwate} was spectroscopically indentical with that derived from O_2Hb A. Motokawa and his coworkers[20] converted metHb M_{Iwate} into hemichrome-like compound in the presence of sodium benzoate. The hemichrome thus prepared could not be distinguished from that of normal hemoglobin by spectroscopic examination. These observations afford evidence for the view that heme is chemically normal in Hb M_{Iwate}. Similar investigation was carried out with other Hb M's; the alkali hemochromes of Hb M_{Osaka} and Hb M_{Akita} were identical with normal alkalihemochrome.[39]

(10) Difference spectrum of Hb M's. As has been discussed in the paragraphs (3) and (5) there is some indication that the molecule of Hb M is composed of two kinds of moieties which are different in ligand reactivity, namely the reactive

and the less reactive subunits. Presumably the reactive subunit will be the normal chain and the less reactive one will represent the abnormal chain. It is expected that the normal chain may show the same absorption spectrum as that of Hb A, but the abnormal chain may exhibits an abnormal light absorption different from that of Hb A, and the spectrum of the abnormal hemoglobin (Hb M) is the result of the summation of light absorption by these two sorts of subunits. Under this assumption difference spectra of Hb M's will be prepared by subtracting the light absorption by the normal subunit (equaling to half as large as that exerted by Hb A of the same concentration) from the absorption curves of the relevant Hb M's. The absorption curves thus obtained are called difference spectra, and these are thought to be representative of the color (or absorption spectra) of the abnormal subunits of Hb M's.

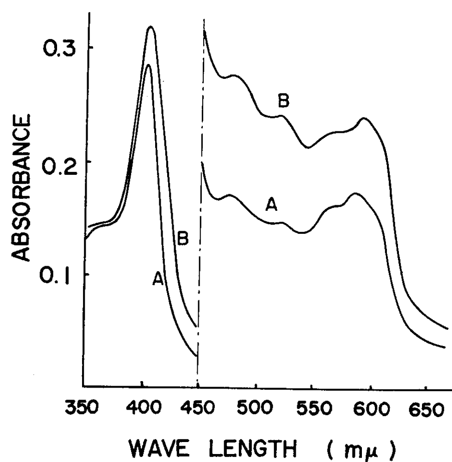

Fig. 15. Difference spectra of Hb M_{Iwate} referring to its abnormal α subunit. (from oxyhemoglobin and methemoglobin)
A: from O_2Hb M_{Iwate}. B: from metHb M_{Iwate}.

The difference spectra of Hb M_{Iwate}, and Hb M_{Akita} are presented in Fig. 15, 16 and 17. It is worthy of special mention that, in Hb M_{Iwate} and Hb M_{Osaka}, the difference spectra of the same shape was obtained from any one of O_2Hb, metHb and cyanmetHb, irrespective of the kind of derivatives.[42] On the contrary, in Hb M_{Akita}, the difference spectra vary according as its derivatives are different.[39] This suggests that the abnormal subunits of Hb M_{Iwate} and Hb M_{Osaka} are chemically inert, being little affected by ligands (including oxygen, cyanide and so forth), whereas in Hb M_{Akita} the abnormal subunit is reactive with ligands to a certain extent, being capable of forming varied complexes of different light absorption. It is very interesting that the Hb M's of Japanese may be classified into two groups: (1) Hb M_{Iwate} and Hb M_{Osaka} wth inert abnormal subunit and (2) Hb M_{Akita} and Hb M_{Kurume} with reactive abnormal subunit.

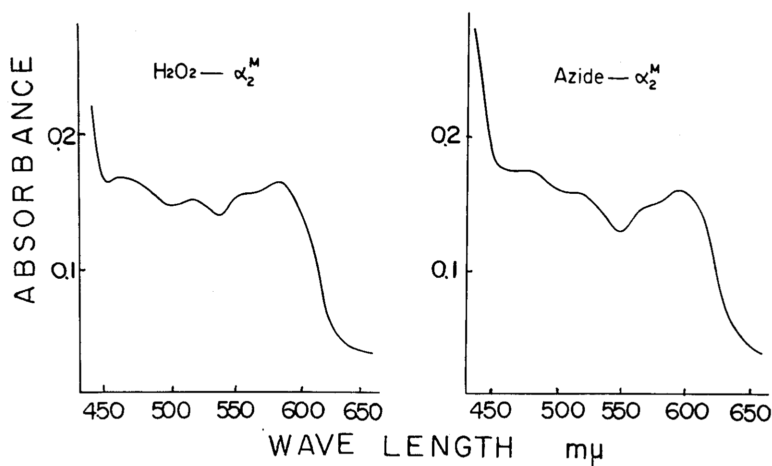

Fig. 16. Difference spectra of Hb M_{Iwate} referring to its α subunit (from H_2O_2-methemoglobin and azide methemoglobin).

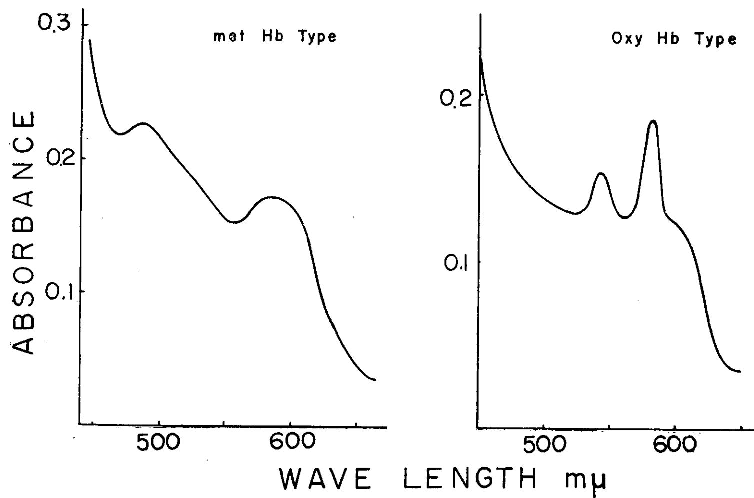

Fig. 17. Difference spectra of Hb M_{Akita} referring to its abnormal β subunit (from methemoglobin and oxyhemoglobin).

Fig. 18. Hybridization of Hb M_{Iwate} (Hb M_I) with canine hemoglobin (Hb Can). $\alpha_2^{Can}\beta_2^A$, $\alpha_2^A\beta_2^{Can}$ and $\alpha_2^M\beta_2^{Can}$ are hybrid hemoglobins. Note that the hybrid hemoglobin $\alpha_2^M\beta_2^{Can}$ is as black as Hb M_{Iwate}, while the hybrid hemoglobin $\alpha_2^{Can}\beta_2^A$ is as red as Hb A and Hb can.

Fig. 27 A hand of the patient with Hb M_{Akita} disease in comparison with that of a normal subject. Left: Normal subject. Right: Hb M_{Akita} disease. Note the cyanotic nails of the fingers of the patient.

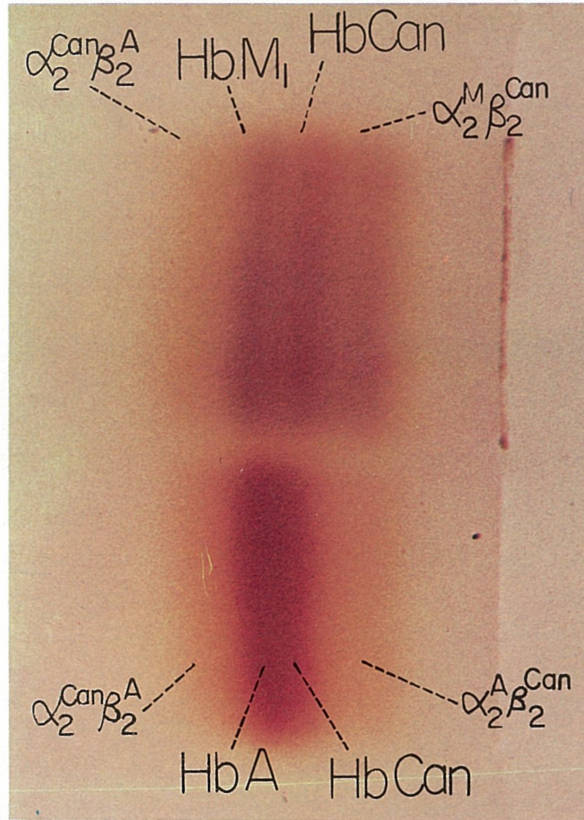

Fig. 18.

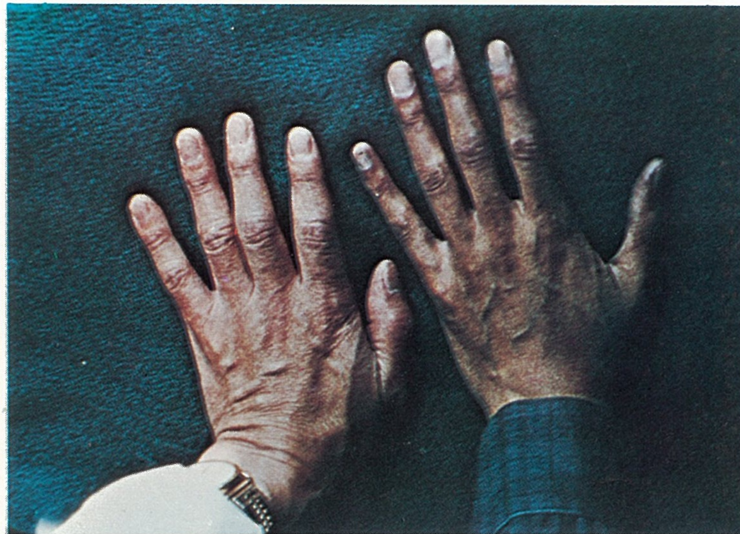

Fig. 27.

ABNORMAL POLYPEPTIDE CHAINS

It was not so easy a task to detect the abnormal polypeptide chains of Hb M's of Japanese. At present a variety of procedures are available for the determination of abnormal chains. They may be classified into three groups: — (1) demonstration of a chain with abnormal electric charge by paper or starch gel electrophoresis of the globin of a purified abnormal hemoglobin after it has been dissociated into its elementary (α and β) chains in a medium with high concentration of urea,[46][47] (2) starch gel electrophoresis of hemolysate containing abnormal hemoglobin together with Hb A after preliminary treatment with parachloromercuribenzoic acid,[48] and (3) agar gel or starch gel electrophoresis of the hybridization product[49] of the purified abnormal hemoglobin with other abnormal hemoglobin of known chain anomaly or with canine hemoglobin.[50]

Lehmann who noticed the presence of Hb A_2 α M_{Iwate} (a fast-moving variant of Hb A_2 consisting of the α chain of Hb M_{Iwate} and δ chain of Hb A_2, i. e. $\alpha_2^M \delta_2^{A_2}$) by paper electrophoresis of the patient's hemolysate suggested for the first time that Hb M_{Iwate} might be an abnormal hemoglobin of α chain anomaly.[18] This was confirmed by Shibata and his colleagues[34][50] by urea dissociation paper electrophoresis of the globin of purified Hb M_{Iwate} as well as by hybridization test of the purified O_2Hb M_{Iwate} with canine hemoglobin. In hybridization test the electrophoretic stripe in starch gel representing the hybrid hemoglobin $\alpha_2^M \beta_2^{Can}$ (composed of the α chain of Hb M_{Iwate} and β chain of canine hemoglobin) migrated to the cathodic extreme and appeared as black as the stripe of the original Hb M_{Iwate}. On the contrary, the hybrid hemoglobin $\alpha_2^{Can} \beta_2^A$ (consisting of the α chain of canine hemoglobin and the β chain of Hb M_{Iwate}) moved to the anodic extreme, being as red as the canine hemoglobin ($\alpha_2^{Can} \beta_2^{Can}$). (Fig. 18). This finding is interpreted that in Hb M_{Iwate} the α chain is abnormal with "black color" while the β chain is normal with red color just like the normal hemoglobin.[51]

Incidentally, it is mentioned that Iuchi and his associates[51] were successful in demonstrating the abnormality of globin in Hb M_{Iwate} by a reconstitution experiment, in which globin of purified Hb M_{Iwate} was coupled with hemin prepared from Hb A to regenerate original metHb M_{Iwate}.

Similar hybridization test examined with respect to other Hb M's unravelled α chain anomaly in Hb M_{Osaka}[30] and β chain anomaly in Hb M_{Kurrme}[32] and Hb M_{Akita}.[32]

It is apparent that the abnormal chains of these Hb M's are "black" in color, being consistent with the absorption curves represented by the relevant difference spectra which were described in the preceding section.

Anyway, Hb M's of Japanese are classified by chain anomaly into two groups:

(1) Hb M_{Iwate} and Hb M_{Oaka} possessing abnormal α chain ($\alpha_2^M\beta_2^A$), and (2) Hb M_{Kurume} and Hb M_{Akita} having abnormal β chain ($\alpha_2^A\beta_2^M$).

FINGERPRINT

Conventional fingerprinting of the tryptic digest of globins[52] are useful to unravel the abnormal tryptic peptide spots in Hb M_{Iwate}, Hb M_{Osaka} and Hb M_{Kurume}, but aminoethylation of globin as preliminary for tryptic digestion was necessary for the demonstration of abnormal spot in the fingerprint of Hb M_{Akita}.[32]

Gerald and Efron[17] were the first who pointed out the exact localization of the abnormal tryptic peptide in the α chain of Hb M_{Iwate}. They noticed that spot No. 3 (α Tp-9) seen on Baglioni's fingerprint[53] gave positive reaction to tyrosine in Hb M_{Iwate}, whereas the same spot was negative for this amino acid in Hb A. Shortly afterwards Miyaji and his associates[54] were successful in isolating clearly the abnormal No. 3 spot (α Tp-9) on the fingerprint by Baglioni's[53] as well as Ingram's[52] technique (Fig. 19). The spot was positve for tyrosine as well as for histidine.

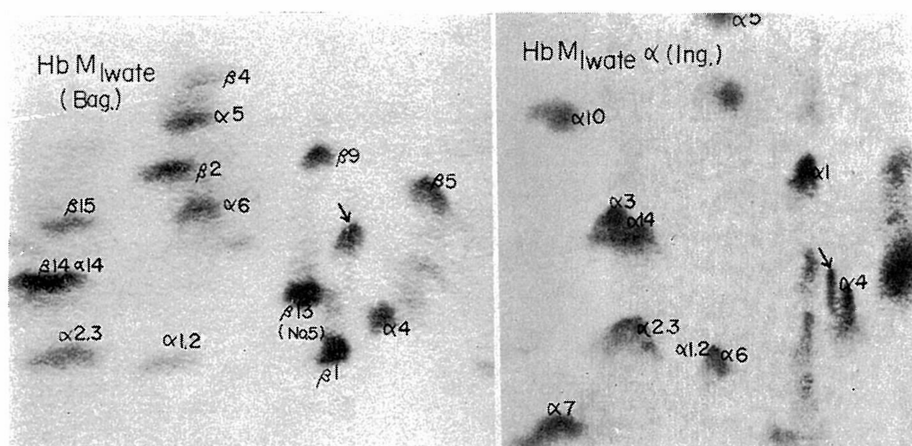

Fig. 19. Fingerprint of Hb M_{Iwate}. Left: Baglioni's technique. Right: Ingram's technipue. Numerals (α 5, β 15 and No. 5, as for instance) refer to tryptic peptides (α Tp-5 and β Tp-15) and Ingram's peptide spots (peptide No. 5). Arrows indicate abnormal peptide spots.

Shibata and others[29] separated β chain from the globin of Hb M_{Kurume} purified by starch block electrophoresis for the purpose of fingerprinting (of Ingram[52] and Baglioni).[53] An abnormal spot made its appearance below spot No. 16 (Fig. 20). The spot was psoitive for tyrosine, negative for histidine, and its eluate resembled β Tp-7 in amino acid composition. In addition, spot No. 20 (β7) was positive for

tyrosine. This spot referring to β Tp-7 should not have tyrosine, if it were of the normal β chain. Detailed examination disclosed it to be peptide β Tp-7, 8 in which histidine residue was substituted for by tyrosine. This abnormal peptide (β Tp-7, 8) happened to occupy the place of the normal spot No. 20, which was really absent on account of the amino acid substitution.

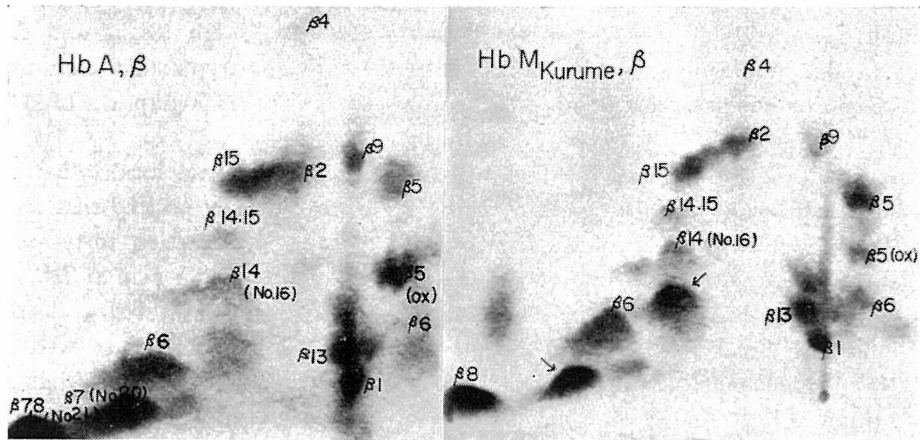

Fig. 20. Fingerprint of Hb M_{Kurume} (by Baglioni's technique). Hb A, β: β chain of Hb A. Hb M_{Kurume}, β: β chain of Hb M_{Kurume}. Arrows indicate abnormal peptide spots. β 5 (ox) refers to oxidized β Tp-5. See the legends for Fig. 19.

Recent study[55] has unravelled on the fingerprint (Baglioni)[53] of Hb M_{Osaka}, an abnormal tyrosine-positive spot partially lapping over the spot No. 17 (Fig. 21).

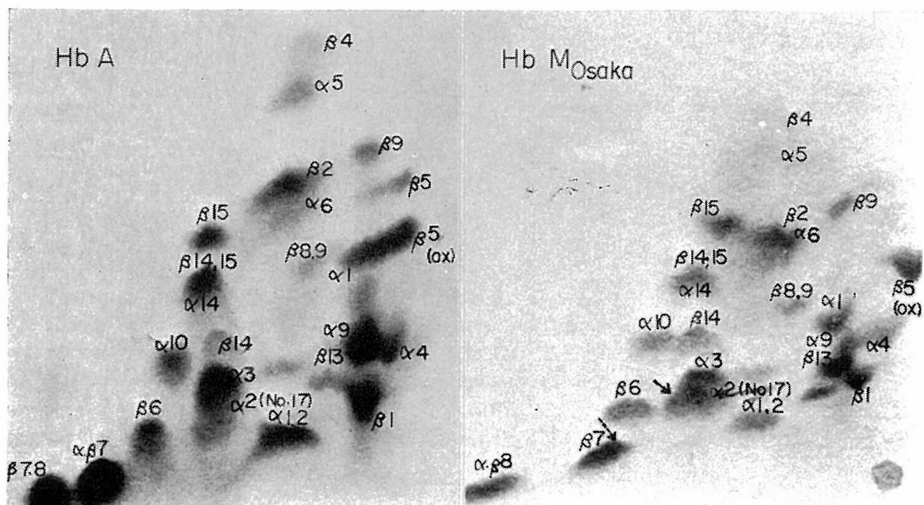

Fig. 21. Fingerprint of Hb M_{Osaka} (by Baglioni's technique). Hb A: fingerprint of Hb A. Hb M_{Osaka}: fingerprint of Hb M_{Osaka}. Arrows indicate abnormal peptide spots. See the legends for Fig. 19.

As stated earlier the conventional fingerprint[52] of the tryptic digest of Hb M_{Akita} was the same as that of Hb A. This is indicative of the abnormal tryptic peptide concealed in the core (the insoluble peptide left insoluble after tryptic digestion). Accordingly the β chain of purified Hb M_{Akita} was prepared, and it was aminoethylated with ethyleneimine so that it might be completely digested by trypsin. The digest of the aminoethylated β chain was fingerprinted by Ingram's[52] procedure. On the peptide map thus made[32] peptide β Tp-10 was not visualized at its proper site ; but an abnormal peptide spot positive for tyrosine and negative for histidine appeared slightly cathode-wards and beside the spot of peptide No. 5 (β Tp-13) (Fig. 22). Another abnormal spot which was similarly positive for tyrosine was seen below the spot No. 5.

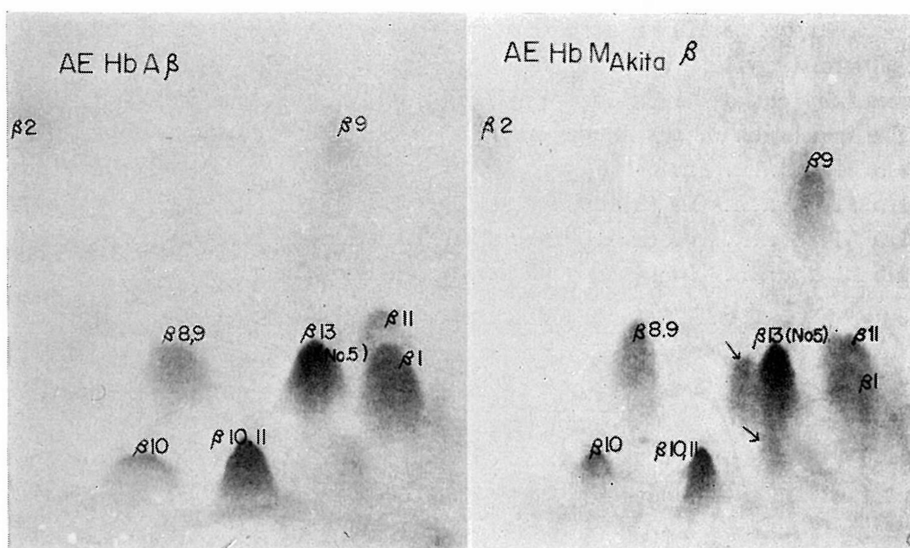

Fig. 22. Fingerprint of aminoethylated globin of Hb M_{Akita}. (Ingram's technique). AE Hb A β: aminoethylated β chain of Hb A. AE Hb M_{Akita} β : aminoethylated β chain of Hb M_{Akita} with contamination of the β chain of Hb A. Arrows indicate the abnormal peptide spot. See the legends for Fig. 19.

To epitomize what has been said above, each of the Hb M's of the Japanese has its own fingerprint, in which one or two peculiar tyrosine-positive abnormal peptide spots are seen. The patterns are listed as below.

Hb M_{Iwate}[54] : ...... An abnormal spot lying obliquely above and to the anode side of spot No. 5 (Ingram and Baglioni).

Hb M_{Osaka}[55] : ...... Two abnormal spots partially lapping over spot No. 17 (Baglioni) and spot β7, respectively.

Hb M_{Kurume}[29] : ......Two abnormal spots visible below spot No. 16 and No. 19 (coincident with spot No. 20), respectively (Baglioni).

Hb M_{Akita}[32] : ...... Two abnormal spots cathodic lateral to and just below spot No. 5 (Ingram ; aminoethylated globin of β chain).

AMINO ACID SUBSTITUTION IN THE ABNORMAL CHAIN

The substitution of amino acid in the abnormal chain of Hb M_{Iwate}[25][26], Hb M_{Kurume}[29] and Hb M_{Akita}[32] was established by Shibata and his associates through amino acid analysis of the eluates of the abnormal spot from the fingerprint. Hb M_{Iwate} was studied also by Konigsberg and Lehmann[19] and Shimizu and his associates.[22] They analyzed the abnormal peptide isolated by column chromatography of the tryptic digest of the globin, and confirmed the validity of Shibata's conclusion.

With respect to Hb M_{Osaka}, Shimizu and his colleagues[31] purified and analyzed its abnormal tryptic peptide in the same way as they did with Hb M_{Iwate}, and successfully elucidated the amino acid substitution in this hemoglobin.

The conclusion of the studies made by these authors were as follows : ——

Hb M_{Iwate} ...... His (α 87) replaced by tyrosine
Hb M_{Osaka} ...... His (α 58) replaced by tyrosine
Hb M_{Akita} ...... His (β 92) replaced by tyrosine
Hb M_{Kurume}...... His (β 63) replaced by tyrosine

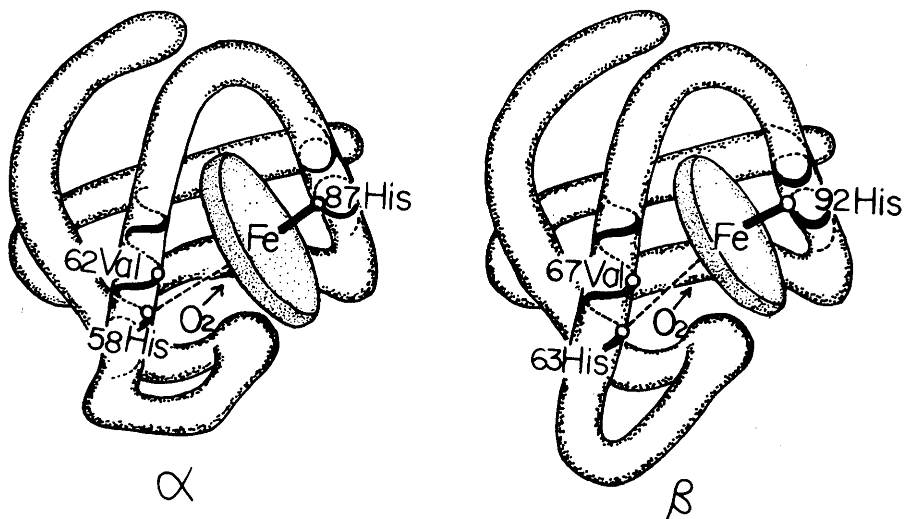

Fig. 23. Three dimensional model of the α and the β chain of hemoglobin. Histidine residues, (α 87 and β 92) and (α 58 and β 63), are proximal and distal histidines of the α or β chain. Fe refers to heme iron of the chain.

Fig. 23 illustrates the site of amino acid substitution in each Hb M. 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 hystidyl residues, the proximal (α 87, β 92) and the distal (α 58, β 63). The proximal histidine acts the role of holder of heme iron, securing to fix heme to the relevant chain, while the distal histidine prevents heme iron from auto-oxidation, enabling hemoglobin molecule to combine with oxygen molecule reversibly.[56][57]

Accordingly, the studies which have been mentioned above reveals that Hb M's of Japanese include all of the variants involving the replacement of the proximal or distal histidine by tyrosine.

FUNCTIONAL ALTERATION OF HEMOGLOBIN MOLECULE

The transportation of oxygen by the blood circulating from the lungs to the tissues owes mainly to the ability of hemoglobin to combine with oxygen reversibly, The degree of combination or of its reversal, i. e., dissociation of oxyhemoglobin to release oxygen is determined by the tension of the oxygen in the medium surrounding the hemoglobin. Normally, at a O_2 tension of 100 mm Hg or more, hemoglobin is completely saturated.

The important relationship between the oxygen saturation of hemoglobin and the oxygen tension is perceived by an examination of the oxygen dissociation curve of hemoglobin, in which the percent saturation is plotted against the oxygen tension. The dissociation curve is sigmoid in shape. This indicates that hemoglobin is oxygenated in a stepwise manner on account of the interaction of hemes belonging to individual α and β chains contained in the molecule. The chain oxygenated earlier makes the next chain take oxygen more easily. Hemoglobin is readily saturated with oxygen, being converted into oxyhemoglobin, in the capillary of the lungs where oxygen tension is around 100 mm Hg, releases oxygen slowly as the oxygen tension drops to about 50 mm Hg, at which point the dissociation curve forms a distinct curvature, and in the tissue, where the oxygen tension is about 40 mm Hg, rapidly dissociates oxygen to make it available to cells.

Carbon dioxide (CO_2) exerts influence upon the oxygen dissociation curve of hemoglobin by lowering pH of the medium. The increase in acidity of the environment apparently facilitates the release of oxygen from oxyhemoglobin, and shifts the slope of the oxyhemoglobin dissociation curve to the right. This phenomenon is referred to as Bohr effect.

As early as in 1962, during the course of their study on the chemistry of Hb M_{Iwate} Shibata and his colleagues[39] were confronted with an incomprehensible observation that this hemoglobin, when aerated, could not carry more than 30 per cent amount of oxygen calculated from the hemoglobin concentration. They hesitated to publish the result because they thought that they might be wrong.

Kikuchi, Hayashi and Tamura[21] (1964) were the first who undertook a systematic study on the oxygenation of the Hb M's of Japanese. They examined the oxygen dissociation curve and the Bohr effect of Hb M_{Iwate}. According to their report this hemoglobin (pH 7.0–7.5) was oxygenated to only approximately 50 and 65 per cent at 50 and 100 mm Hg of pO_2, respectively, whereas Hb A (pH 7.0–7.5) was saturated to almost 100 per cent under these oxygen tensions. The oxygen dissociation curve of Hb M_{Iwate} was significantly less sigmoid than that of Hb A (In Hb M_{Iwate} the n value for Hill's equation was 1.0 in contrast to 2.8 in Hb A). Hb M_{Iwate} showed no Bohr effect in the pH range of 6.3 to 7.5, although its slight indication was noted above pH 7.5 (The maximum value of $\Delta \log pO_2 / \Delta pH$ was about 0.19 in Hb M_{Iwate}, and approximately 0.50 in Hb A). (Fig. 24). These observations indicate that Hb M_{Iwate} is distinctly inferior to Hb A in oxygen affinity, and it is far less effective in oxygen transportation than Hb A.

Similar lowered oxygen affinity (one seventh as much as that of Hb A) was found in Hb M_{Osaka} by Suzuki and others.[58] Scrutiny of the oxygen dissociation curve depicted in their paper tells us that Hb M_{Osaka} (pH 6.49–7.89) is oxygenated to approximately 55 and 75 per cent at pO_2 of 50 and 100 mm Hg, respectively. (The oxygen dissociation curve was almost rectilinear in shape with a n value of 1.2 for Hill's equation when per cent saturation was plotted against log pO_2. The Bohr effect was not recongnizable within the pH range from 6.49 to 7.89).

Hb M_{Kurume} was apparently different from Hb M_{Iwate} and Hb M_{Osaka} in the attitude of oxygenation. According to Suzuki and his associates[59] Hb M_{Kurume} was approximately equal to Hb A in oxygen affinity and Bohr effect, although the sigmoidity of dissociation curve was diminished. (At pH 7.3 Hb M_{Kurume} is oxygenated to approximately 70 and 25 per cent at pO_2 of 10 and 2 mm Hg, respectively, while Hb A is saturated with oxygen to 80 and 5 per cent at these oxygen tensions. Hence Hb M_{Kurume} is less efficient in oxygen transportation than Hb A in spite of almost normal O_2 affinity as judged by 50 % saturation of the per cent saturation-pO_2 curve.). (Fig. 25).

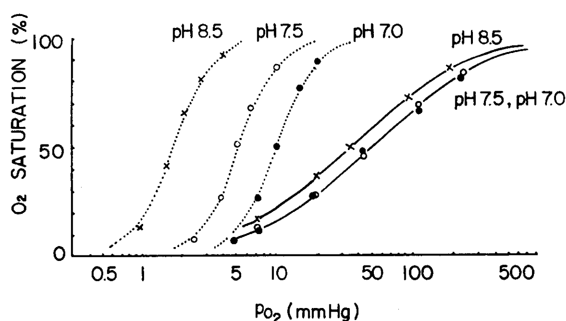

Fig. 24. Oxygen dissociation curve of Hb M_{Iwate} (solid line) in comparison with that of Hb A (dotted line).

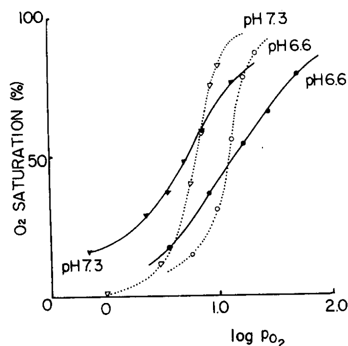

Fig. 25. Oxygen dissociation curve of Hb M_{kurume} (solid line) in comparison with that of Hb A (dotted line).

Recently Morimoto and Imai[60] studied the oxygen dissociation curve of Hb M_{Akita}, and came to the conclusion that this Hb M may be a little superior to Hb A in oxygen affinity and Bohr effect. The dissociation curve, however, was somewhat less sigmoid in shape.

In short, it is worthy of special mentioning that the Hb M's belonging to the group of α chain anomaly (Hb M_{Iwate} and Hb M_{Osaka}) exhibit a remarkable impairment of oxygen transportation in contrast to almost normal or slightly impaired functional capacity of the Hb M's of β chain anomaly (Hb M_{Kurume} and Hb M_{Akita}).

ELECTRON SPIN RESONANCE (ESR)

Hayashi and his associates[61][62] successfully applied ERS for the identification of Hb M_{Iwate}, Hb M_{Osaka} and Hb M_{Kurume}, and quite recently Morimoto and his colleagues[60] studied the ESR of Hb M_{Akita}.

Heparinized blood from the patients (0.2 ml) was placed in a 3 mm quartz tube to be inserted into the resonant cavity of Varian ESR spectrometer. The ESR was measured at liquid nitrogen temperature, and the signal was recorded on the Y-axis of a X-Y recorder. The ESR signals in the region $g \approx 6$ are important because the ESR of Hb M variants revealed remarkable abnormality in this region. Hemoglobin of normal human blood is with ferrous heme iron, so its ESR was not observed. In Hb M of patient's blood the signal was strong at about $g \approx 6.0$ as shown in Fig. 26. According to Hyashi and others[61] this is an evidence of the presence of ferric heme iron in the molecule of Hb M.

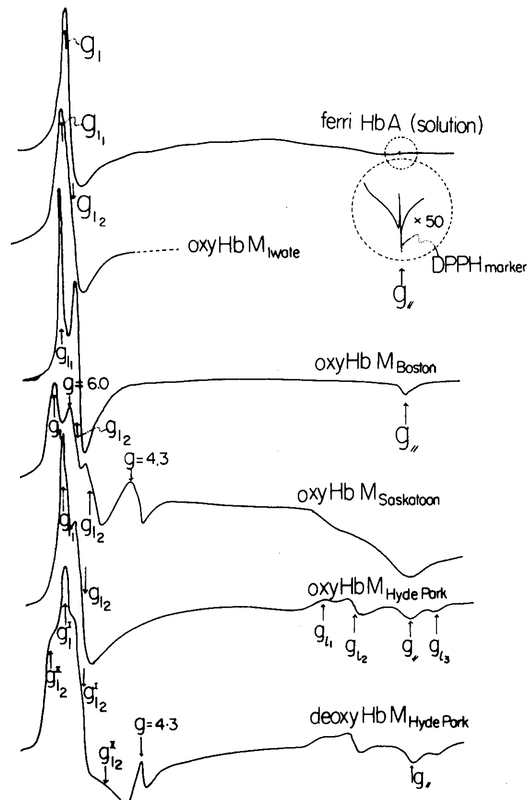

Fig. 26. ESR of Hb M,s of Japanese.

MOLECULAR STRUCTURE AND OXYGEN TRANSPORTATION

As early as in 1959 Gerald and George[45] propounded a theory on the molecular structure of Hb M, which laid the foundation of further development of our knowledge in this field. They thought that the methemoglobin (the abnormal chain) of Hb M would be a chromoproteid whose heme iron was combined with globin (the abnormal chain) by means of its main and side-arm polypeptides as indicated by Globin-F_e^+-Y (Y is the side-arm peptide). Normal methemoglobin is globin-F_e^+-(H_2O), if its chemical structure is shown in the same way. The ligand (L) which attacks heme iron directly is able to break the combination of heme iron with side arm peptide, resulting in a compound Y-Globin-Fe-L. This reaction will be rapid or slow according as the affinity of side arm peptide to heme iron is weak or strong.

As stated earlier, it is inferred from the results obtained by the spectroscopy of deoxygenated hemoglobin and cyanmethemoglobin of the Hb M's of Japanese that the molecules of these hemoglobins are composed of two sorts of moieties, namely the subunit with ferrous heme iron and that with ferric heme iron. ESR study provides further evidence in support of the presence of ferric heme iron in the Hb M's. It has been known that proximal and distal histidines of the polypeptide chains of hemoglobin prevent the heme iron from autoxidation (methemoglobinization), and this mechanism is expected to be invalidated in the Hb M's of Japanese on acount of the presence of tyrosine in substitution for either one of these histidine residues. It is therefore easily conceivable that this ferric heme iron belongs to the abnormal chain, but not to the normal chain.

In addition, it will be germane to consider that the tyrosine which is in substitution for proximal or distal histidine will constitute an important part of the side-arm peptide of Gerald and George.[45] In fact, the tyrosine alone is thought to make all of the side-arm peptide, because amino acid analysis fails to disclose the difference between the abnormal and the normal chains except at the proximal or distal histidine. According to Ingram[63] in the abnormal chain of Hb M the phenol side chain of the tyrosine (in substitution for histidine) comes in right position to bond to ferric heme iron (the sixth coordinate position) as an internal ligand, forming a phenolate complex.

It is inferred from the study of ligand reactivity which has been mentioned above that the phenolate complex is very stable in Hb M_{Iwate} and Hb M_{Osaka}, rejecting every sort of ligands including oxygen, cyanide, fluoride, dithionite and azide. Neither is it affected by DPNH and TPNH methemoglobin reductases which serve as a reducing agent to spontaneously produced intraerythrocytic methemoglobin. The fact that the difference spectra of the abnormal chain of Hb M_{Iwate} remain the same even if its derivatives are changed by addition of

various ligands is the evidence for the stable phenolate complex in the abnormal chain of this hemoglobin. It is accordingly presumable that in Hb M_{Iwate} and Hb M_{Osaka} half a molecule corresponding to the abnormal α chains is incapable of oxygen transportation and only the normal β chains forming the remaining part of the molecule carry oxygen. However, the deleterious effect is not restricted in the realm of a half molecule. It involves also the impairment of oxygenation of the normal β chain, invalidating the heme-heme interaction and α-β interaction for Bohr effect due to inertness of the α chains. It is speculated that when the molecule of hemoglobin is oxygenated the α chains combine with oxygen first and then the β chains are brought into the state of receiving oxygen by the heme-heme and the α-β interaction. In Hb M_{Iwate} and Hb M_{Osaka} their α chains reject oxygen because of their abnormality, and thus render the next step of receiving oxygen into the β chains difficult. This will be the reason why these hemoglobins show pronounced diminution of oxygen affinity and loss of Bohr effect.

In Hb M_{Kurume} and Hb M_{Akita} the α chains are normal, being capable of combining oxygen with their ferrous heme iron. So they would be able to introduce the next step of oxygenation if the β chains were normal. In reality, the chains of these hemoglobins are abnormal and can not carry oxygen on account of the phenolate complex formed between the ferric heme iron and the internal ligand of the tyrosine residue (β 63 or β 92). However, the phenolate complex is not so stable as seen in Hb M_{Iwate} and Hb M_{Osaka}. The ferric heme iron of the abnormal β chains of these hemoglobins is reactive to a certain extent when extrinsic ligand is present in abundance. Even some amount of oxygen may be received reversibly by the abnormal β chain. In this way, the Hb M's of β chain anomaly maintain their oxygen affinity and Bohr effect close to the normal level, although their heme-heme interaction is impaired. It is therefore true that though Hb M's are less effective as vehicle of oxygen transportantion than Hb A, the capacity is not so seriously disturbed in Hb M_{Akita} and Hb M_{Kurume} (of β chain anomaly) as in Hb M_{Iwate} and Hb M_{Osaka} (of α chain anomaly).

Nevertheless, it can not be hastily considered that the Hb M's of β chain anomaly is physiologically more competent than the Hb M's of α chain anomaly. Hb M_{Akita} and Hb M_{Kurume} are labile and prone to auto-oxidation in vivo as well as in vitro, whereas Hb M_{Iwate} and Hb M_{Osaka} are relatively stable. According to Hayashi[64] the β chain plays an important role in securing the integrity of hemoglobin molecule. If this conception is correct, the vulnerability to deterioration in Hb M_{Akita} and Hb M_{Kurume} will be accounted for by the abnormality of their β chains.

GENETICS

An extensive genetic study of hereditary nigremia (Hb M_{Iwate} disease) was

carried out by Tamura and his associates.[4)5)10)11)] They had visited the families of the patients one after another at the spot with perseverance for about ten years, until they were successful in revealing a huge family tree extending over seven generations which included 70 patients (31 males and 39 females) and 292 healthy subjects. According to their field investigation the first authentic patients with hereditary nigremia lived during the period of the latter half of 1800's. Some of their ancestors and their parents were supposed to have been the carriers of this disease.

On the basis of this investigation Tamura came to the conclusion that hereditary nigremia (Hb M_{Iwate} disease) was transmitted as a typical autosomal dominant character with complete penetrance and all the patients so far diagnosed were heterozygous for the gene.

In Hb M_{Osaka} disease a house-wife (aged 41) and one (a boy aged 17) of her three children were cyanotic. Her parents were apparently normal.[30)]

The patient of Hb M_{Kurume} disease was a cyanotic boy (aged 6), his parents were not cyanotic, and all the relatives of patient's family were normal.[27)]

In the family of Hb M_{Akita} disease, the propositus was a 44 year old man whose parents were normal. He had a boy and a girl. Both of the children were cyanotic.[32)]

The genetic aspect of these three Hb M diseases was the same as was in Hb M_{Iwate} disease: The probands were heterozygotes of an autosomal dominant gene for the abnormality, possessing Hb M together with Hb A in blood. Homozygous individuals have never been encountered.

That the parents of the proband was normal in Hb M_{Osaka}, Hb M_{Kurume} and Hb M_{Akita} diseases is strange. This may suggest that Hb M diseases may be arising from the recent mutation of the hemoglobin genes.

COMPARISON WITH THE HB M'S OF THE FOREIGN COUNTRIES

Since Hörlein and Weber[13)] discovered the first instance of Hb M in Elberfeld, Germany, not less than seven variants have been reported from various areas of the world. In early days Hb M disease was thought to be a hemoglobinopathy exclusively partaining to the Caucasians, but it was later found among the yellow races, and quite recently among the negroes, too.

In 1962 Betke[65)] pointed out that in Hb M diseases the absorption curves of the acid metHb type hemolysates were characterized by larger values for quotients* E_{630} / E_{600} and E_{500} / E_{600} exceeding 1.25 and 2.80, respectively. He

*E_{630}, E_{600}, and E_{500} refer to the optical densities (optical path, 1.0 cm) at 630, 600 and 500 mμ, respectively.

classified Hb M diseases into three groups on the basis of the shape of the absorption curve of acid metHb type hemolysate. According to his classification, the curve forms a plateau around 600 mμ in (1) the Boston type, a peak in (2) the Saskatoon type, and a depression in (3) the Milwaukee-1 type. Table II presents the variants of Hb M disease of the world as classified by Betke's principle.

Table II. Hb M's of the world

A. Boston type ...... The absorption curve of the acid metHb type hemolysate shows an almost horizontal plateau, forming neither a peak, nor a depression around 600 mμ (630~550 mμ).

1. Boston[15)45)] ...... (Syn. Gothenburg,[37)] Norin,[75)] Leipzig II,[76)] and Osaka)[31)]
   $\alpha_2^{58Tyr}\beta_2$; its cyanmetHb is different from cyanmetHb A in the shape of absorption curve; metHb 598, 495 mμ ; 22 ~ 42 % ; rarely associated with mild hemolytic anemia ; Germany, Sweden, U. S. A., Japan and England.
2. Iwate[6)25)26)35)] ...... (Syn. Kankakee[66)68)] and Oldenburg)[77)78)]
   $\alpha_2^{87Tyr}\beta_2$; its cyanmetHb is different from cyanmetHb A in the shape of absorption curve; metHb 590, 490 mμ ; 30% ; Japan, U.S.A., Germany, Israel and Switzerland.
3. Hyde Park[70)] ...... (Syn. Akita)[32)]
   $\alpha_2\beta_2^{92Tyr}$; its cyanmetHb is almost the same as cyanmetHb A in absorption curve ; metHb 578, 496 mμ (depression, 560 mμ); 20 ~ 30 % ; U. S. A. and Japan ; Occ. associated with mild hemolytic anemia.
4. Milwaukee-2[17)79)]
   $\alpha_2\beta_2^M$ (together with Hb E); ? ; metHb 605, 495 mμ; its cyanmetHb may be different from cyanmetHb A in absorption curve ? ; U. S A. (Swiss ancestry).
5. Reserve[80)]
   $\alpha_2^M\beta_2$; its cyanmetHb is different from cyanmetHb A in the shape of absorption curve; metHb 605, 500 mμ (depression, 565 mμ, the peaks seem to be more salient than in metHb M_{Iwate}); 28 % ; U. S. A

B. Saskatoon type ...... The absorption curve of the acid methemoglobin type hemolysate shows a small peak or protrusion around 600 mμ.

1. Saskatoon[15)17)83)] .... (Syn. Emory,[17)] Kurume,[27)29)35)] Radom,[81)] Arhus,[82)] Hamburg,[85)] Chicago[86)]).
   $\alpha_2\beta_2^{63Tyr}$; its cyanmetHb is almost the same as cyanmetHb A in the shape of absorption curve ; metHb 600, 540 and 488 mμ (depression, 560 mμ) ; 25 % ; Canada, U. S. A., Japan, Germany and England. Occ. associated with mild hemolytic anemia.
2. Leipzig I[87)] ...... (Syn. Elberfeld,[13)] Göttingen[88)]).
   ? ; metHb 600, 540mμ ? (depression, 560 mμ); metHb reacts with cyanide slowly, but the absorption curve of its cyanmetHb is almost the same as that of cyanmetHb A ; 25 %, Germany.

C. Milwaukee-1 type ...... The absorption curve of the acid metHb type hemolysate shows a depression over the range 630 ~ 550 mμ, being similar to metHb A to a certain extent.

1. Milwaukee-1[17)19)]
   $\alpha_2\beta_2^{67Glu}$; metHb 625, 425 mμ (distinct depression 595 mμ); its cyanmetHb is almost the same as cyanmetHb A in the shape of absorption curve ; U. S. A. (Italian ancestry).

2. Freiburg[89)90)]

$\alpha_2\beta_2^{23Val\to O}$; metHb 630, (560 ~ 570), (530 ~ 540), 500 mμ (depression, 600 mμ); its cyanmetHb is almost the same as cyanmetHb A in the shape of absorption curve; MetHb M Freiburg is not separable from metHb A by electrophoresis at pH 7.0; CyanmetHb M Freiburg is separated from cyanmetHb A by electrophoresis at pH 8.6 (between cyanmetHb A and cyanmetHb F); associated with mild hemolytic anemia.

....

Hb M's of obscure character:

Kiskunhals,[91)] Cologne,[92)] Budapest[93)] and Paris.[94)]

....

Explanation for reading this table: — "metHb 600, 495 mμ" means that methemoglobin derivative has two peaks at 600 mμ and 495 mμ. "22-42 %" refers to that the content of the relevant Hb M is from 22 to 42 per cent of the total hemoglobin contained in hemolysate.

"Germany. Sweden, U. S. A., Japan and England" indicates that the relevant Hb M was detected from the people living in Germany, Sweden, etc.

It will be apparent from this table that Hb M_{Osaka}, Hb M_{Iwate}, Hb M_{Kurume} and Hb M_{Akita} do not belong to Japanese exclusively, but they are shared with by other races.

Hb M_{Iwate} was detected in Chicago by Heller and his associates[66)] two years after its discovery in Japan. It was named tentatively Hb $M_{Kankakee}$. The identity of Hb $M_{Kankakee}$ with Hb M_{Iwate} was established in the laboratories of Heller and Shibata by spectroscopic examinations of the cross-exchanged samples of the patient's blood.[67)] Jones and his colleagues[68)] confirmed that Hb $M_{Kankakee}$ is $\alpha_2^{87Tyr}\beta_2^A$ by amino acid analysis of the abnormal tryptic peptide. This hemoglobin is distributed in Japan, U. S. A., Germany, Israel and Switzerland.[67)69)]

Hb M_{Osaka} is identical with Hb M_{Boston} which was discovered by Gerald[15)] in Boston in 1948. It is seen among the peoples of the U.S.A., Germany, Sweden, Japan and England.[67)69)]

Hb M_{Kurume} is the same as Hb $M_{Saskatoon}$ which was discovered in Saskatoon, Canada, by Baltzan[83)] in 1950. This hemoglobin has many synonyms (Hb M_{Emory}, Hb M_{Radom}, Hb M_{Arhus}, Hb $M_{Chicago}$ and Hb $M_{Hamburg}$) and shows a wide distribution covering over the countries including Canada, Japan, Germany and England.[67)69)]

Hb M_{Akita} is identical with Hb $M_{Hyde\ Park}$ which was discovered by Heller and others[70)] in U. S. A. in 1966. This is worthy of special mentioning as the first instance of Hb M that has ever been detected from negro.

SYMPTOMATOLOGY

Hemoglobin M diseases of Japanese are classified into two groups, namely (1) conginital cyanosis without causing deleterious effect on daily life, and (2)

congenital cyanosis with mild hemolytic anemia. Hb M_{Iwate} disease and Hb M_{Osaka} disease in which abnormal hemoglobin of α chain anomaly is produced belong to the group (1), while Hb M_{Akita} disease and Hb M_{Kurume} disease which yield the abnormal hemoglobin of β chain anomaly pertain to the group (2). The sign of increased hemolysis will be accounted for by shortening of the life span of erythrocytes containing vulnerable Hb M's of β chain anomaly. The Hb M's of α chain anomaly is stable. Therefore, life span of erythrocytes is expected to be normal in Hb M_{Iwate} disease and Hb M_{Osaka} disease. It is also said that the patient is born as a cyanotic baby in the Hb M diseases of α chain anomaly, whereas in Hb M diseases of β chain anomaly cyanosis does not appear until the second year (or later) after birth when Hb A ($\alpha_2^A \beta_2^A$) becomes predominent over Hb F ($\alpha_2 \gamma_2$) completely.[71]

Hb M disease may be frequently confused with congenital heart disease and hereditary methemoglobinemia. In Hb M disease clubbed (drumstick) fingers which are common in congenital heart disease is not observed, and administration of large dose of ascorbic acid and intravenous injection of methylene blue which are beneficial for hereditary methemoglobinemia are not effective measures for the treatment of Hb M diseases.[71]

The diagnosis of Hb M diseases of Japanese is established by spectroscopic examination of the acid metHb type hemolysate[6] or by agar gel electrophoresis (pH 7.0)[6][34] of the hemolysate (O_2Hb type or metHb type). Conventional hematological examination is also helpful for the Hb M diseases of β chain anomaly. There is no defective activity of the intraerythrocytic enzymes.[4]

Group (1) : — Hb M_{Iwate} disease is the representative example. The countenance of the patient is reminiscent of a man who has come out of chilly water after swimming for a long time : The lips, cheeks and auricles are cyanotic. The oral mucosa (gingiva and tongue), and the nail bed of the fingers are also pale and blue violet. There is no deformation of the tips of the fingers and toes. The glans penis (man), the external genitalia (woman) and the orificium uteri (woman) are dark, being almost black. The fundus of the eye is dark and it is hard to discriminate retinal arteries from veins by fundoscopy, because both are black in color. Apart from these clinical manifestations related to cyanosis, the patient enjoys a healthy life and normal physical activity. One of the patients proved to be a competent soldier in military service. He was nicknamed "crow-sergeant," because he was black and strong.[4]

Physical examinations including those for circulatory disturbances are entirely negative.[27] Conventional hematological tests are whthin normal range, although slight polycythemia may be seen occasionally.[4]

Group (2) : — In Hb M_{Akita} patient[72] the facial complexion, lips cheeks auricles, gingiva, tongue, hand and palm are livid in color. Conjuctiva palpebrae is with blackish·tint. The chest is clear to percussion and auscultation. The

liver is not palpable, but the spleen is enlarged just below the costal arch. The urine is postive for urobilinogen. The erythrocyte count is around the lower limit of normal range (The cells are normocytic and normochromic). Diffusely basophilic cells are relatively frequent with slight increase in reticulocyte count (about 3 per cent) in the Wright-stained blood smear. Heinz body test with acetylphenylhydrazine[73] is slightly positive. Plasma color is icteric (serum bilirubin is around 1.5 mg/dl. The direct bilirubin is less than 50 per cent of the total bilirubin).

According to Kimura and his colleagues[27] no sign of increased hemolysis was observed in the patient with Hb M_{Kurume} disease, but it is suspected that he may on occasions have very mild hemolytic tendency.

SUMMARY

Hb M diseases are the most important hemoglobinopathy in Japan. They are Hb M_{Iwate} disease (in Iwate prefecture and Hokkaido), Hb M_{Boston} disease (in Osaka), Hb $M_{Hyde\ Park}$ disease (in Akita prefecture) and Hb $M_{Saskatoon}$ disease (in Hida, Ooita prefecture). The hemoglobins of these diseases cover all the variants of Hb M's brought about by the replacement of the proximal or the distal histidine by tyrosine.

The presence of these hemoglobins in blood is demonstrated by the spectroscopic examination of acid metHb type hemolysate, whose absorption curve lacks depression around 600 mμ, which is characteristic of the normal acid methemoglobin.

In the methemoglobin derivatives of the Hb M's pertaining to the amino acid substitution at proximal histidine (Hb M_{Iwate} and Hb M_{Akita}) the second peak of their absorption curves (around 500 mμ) is shifted to the side of shorter wave length in comparison with that in the Hb M's relevant to the substitution of the distal histidine (Hb M_{Osaka} and Hb M_{Kurume}).

Cyanmethemoglobin of the Hb M's of α chain anomaly (Hb M_{Iwate} and Hb M_{Osaka}) shows an absorption peak (around 540 mμ) with wide base which is distinctly different in shape from that of cyanmetHb A. By contrast, the absorption curve of the Hb M's of β chain anomaly (Hb M_{Akita} and Hb M_{Kurume}) is almost the same as that of cyanmetHb A.

The reactivity of methemoglobins with sodium dithionite is slow and incomplete in the Hb M's of α chain anomaly (Hb M_{Iwate} and Hb M_{Osaka}) and rapid and nearly complete in the Hb M's of β chain anomaly (Hb M_{Akita} and Hb M_{Kurume}).

The Hb M's of α chain anomaly are easily demonstrated by direct application of hemolysate (O_2Hb type) to agar gel electrophoresis, but those of β chain anomaly are seldom detectable by this technique unless the hemolysates have been treated preliminarily with ferricyanide.

The Hb M's of α chain anomaly (Hb M_{Iwate} and Hb M_{Osaka}) cause cyanosis but they are without any significant ill effect on health. On the contrary, those of β chain anomaly (Hb M_{Akita} and Hb M_{Kurume}) may bring about clinical manifestations of mild hemolytic anemia in addition to cyanosis.

REFERENCES

1) Shibata, S.: Hemoglobinopathy, with special reference to the abnormal hemoglobins found in Japan. *Acta Haem. Jap.*, **24**: 141-155, 1961 (in Japanese).
2) Shibata, S.: Hemoglobinopathy, with special reference to the abnormal hemoglobins found in Japan. *Bull. Yamaguchi Med. School,* **8**: 197-207, 1961.
3) Shibata, S., Iuchi, I., and Miyaji, T.: Abnormal hemoglobins discovered in Japan. *Acta Haem. Jap.*, **29**: 115-127, 1966.
4) Tamura, A., Takahashi, M., Ishii, H., and Akashi, S.: A study on the hereditary black blood disease. *Jap. J. Human Genet.*, **4**: 180-195, 1959.
5) Tamura, A.: Black blood disease (A prize winning dissertation at the 9th Annual Congress of the Japan Society of Human Genetics). *Jap. J. Human Genet.*, **9**: 183-192, 1964.
6) 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.
7) Kimura, K., and Sasaki K.: A hereditary disease with blackish brown blood as a principal clinical manifetastion. *Tokyo Iji-shinshi*, No. 2978 : 1158, 1936 (in Japanese) Quoted by[4] Tamura et al.
8) Shikinami Y., and Hosokawa, H.: Hereditary Addison's disease. *Tohoku Igakkai-Zasshi,* **20**: 8, 1937. Quoted by 4) Tamura et al.
9) Tanobe, T.: Ophthalmological symptoms of a patient of hereditary anomaly with blackish brown blood. *Nippon Ganka-gakkai Zasshi,* **45**: 105, 1941, Quoted by 4) Tamura et al.
10) Tamura, A., and Takahashi, M.: Study on the hereditary black blood disease (Tamura and Takahashi's disease). *Tohoku J. Exp. Med.*, **54**: 209-213, 1951.
11) Tamura, A., Takahashi, M., Ishii, H., and Akashi, S.: Study on Tamura and Takahashi's disease. *Tohoku J. Exp. Med.*, **64**: 333-341, 1956.
12) Akashi, S.: Studies on hereditary nigremia (Tamura-Takahashi's disease). *Nagoyaigaku,* **74**: 1007-1017, 1957.
13) Hörlein, H., und Weber, G.: Über chronische familiäre Methämoglobinämie, und eine neue Modifikation des Methämoglobins. *Deut. Med. Wschr.*, **73**: 476-478, 1948.
14) Singer, K.: Herditary hemolytic disorders associated with abnormal hemoglobins. *Amer. J. Med.*, **18**: 633-652, 1955.
15) Gerald, P. S.: The electrophoretic and spectroscopic characterization of Hgb M. *Blood*, **13**: 936-949, 1958.
16) Tamura, A.: Black blood disease. *Shinryo,* **16**: 212-216, 1963. (in Japanese)
17) Gerald, P. S., and Efron, M. L.: Chemical studies of several varieties of Hb M. *Proc. Nation. Acad. Sci.*, **47**: 1758-1767, 1961.
18) Lehmann, H. Haemoglobins and haemoglobinopathies. A review of recent abvances. Lehmann, H., and Betke, K.: Haemoglobin Colloquium. Thieme (Stuttgart), 1962. (pp. 1-14).
19) Konigsberg, W., and Lehmann, H.: The amino acid substitution in hemoglobin M_{Iwate}. *Biochim. Biophys. Acta,* **107**: 266-269, 1965.
20) Motokawa, Y., Hayashi, N., and Kikuchi, G.: On the reactivity of hemoglobin M_{Iwate}. *Arch. Biochem. & Biophys.*, **105**: 612-619, 1964.
21) Kikuchi, G., Hayashi, N., and Tamura, A.: Oxygen equilibrium of hemoglobin M_{Iwate}. *Biochim. Biophys. Acta,* **90**: 199-201, 1964.

22) Shimizu, A., Tsugita, A., Hayashi, A., and Yamamura, Y.: The primary structure of hemoglobin M_{Iwate}. *Biochim. Biophys. Acta,* **107**: 270-277, 1965.
23) Shibata, S., Tamura, A., Iuchi, I., and Miyaji, T.: Hereditary nigremia and hemoglobin M_{Iwate}. *Proc. Jap. Acad.,* **40**: 220-225, 1964.
24) Shibata, S.: Hereditary nigremia (geneticobiochemical aspects). (A prize winning dissertation at the 9th Annual Congress of the Japan Society of Human Genetics). *Jap. J. Human Genet.,* **9**: 193-206, 1964.
25) 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.
26) Miyaji, T., Iuchi, I., Shibata, S., Takeda, I., and Tamura, A.: Possible amino acid substitution in the α chain (α^{87Tyr}) of Hb M_{Iwate}. *Acta Haem. Jap.,* **26**: 538-543, 1963.
27) 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.
28) Yamaoka, K.: Hemoglobinopathy in Kyushu. *Shinryo,* **16**: 181-197, 1963 (in Japanese).
29) 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.
30) 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.
31) 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.
32) Shibata, S., Miyaji, T., Karita, K., Iuchi, I., Ohba, Y., and Yamamoto, K., A new type of hereditary nigremia. discovered in Akita—Hb $M_{Hyde\ Park}$ disease.*Proc. Jap. Acad.,* **43**: 65-70, 1967.
33) Shibata, S., and Iuchi, I.: A simple technique of agar gel electrophoresis for rapid separation of hemoglobins. *Acta Haem. Jap.,* **24**: 51-58, 1961.
34) Shibata, S., Miyaji, T., Iuchi, I., and Ueda, S.: A comparative study of hemoglobin M_{Iwate} and hemoglobin M_{Kurume} by means of electrophoresis, chromatography and analysis of peptide chains. *Acta Haem. Jap.,* **24**: 486-494, 1961.
35) Shibata, S., Iuchi, I., et Miyaji, T.: L' hémoglobinose M au Japon. *Nouv. Rev. Fr. d'Hé mat.,* **5**: 49-62, 1965.
36) Obara, K., Oikawa, M., Fujita, K., and Kuzuya, H.: Paper electrophoretic pattern of hereditary nigremia erythrocytes. *Acta Haem. Jap.,* **25**: 739-742, 1962.
37) Hansen, H. A., Jagenburg, O. R., and Johansson, B. G.: Studies on an abnormal hemoglobin causing hereditary congenital cyanosis. *Acta Paediat.,* **49**: 503-511, 1960.
38) Huisman, T. H. J., and Prins, H. K.: The chromatographic behaviour of different abnormal human haemoglobins on the cation exchanger Amberlite IRC 50. *Clin. Chim. Acta,* **2**: 307-311, 1957.
39) Shibata, S., and Juchi, I.: Unpublished data.
40) Shibata, S., Iuchi, I., Miyaji, T., and Ueda, S.: Spectroscopic characterization of Hb M_{Iwate} and Hb M_{Kurume}, the two variants of hemoglobin M found in Japan. *Acta Haem. Jap.,* **24**: 477-485, 1961.
41) Shibata, S., Iuchi, I., Takeda, I., and Tamura,A.: Spectrophotometric determination of Hb M_{Iwate} in the hemolysate of hereditary nigremia. *Acta Haem. Jap.,* **26**: 159-163, 1963.
42) Shibata, S., Iuchi, I., Miyaji, T., and Tamura, A.: Molar extinction coefficients of hemoglobin M_{Iwate} with a note on the spectrophotometric determination of this hemoglobin in the hemolysate of hereditary nigremia. *Acta Haem Jap.,* **26**: 641-649, 1963.
43) Hunter, F. T.: The quantitation of mixtures of hemoglobin derivatives by photoelectric spectrophotometry. Thomas (Springfield), 1951.
44) Shibata, S., and Iuchi, I.: unpublished data.
45) Gerald, P. S., and George, P.: Second spectroscopically abnormal methemoglobin associated with hereditary cyanosis. *Science,* **129**: 393-394, 1959.

46) Take, T.: On the dissociation of hemoglobin under the action of urea. *J. Biochem.*, **49**: 206-210, 1961.
47) Chernoff, A. I.: The amino acid composition of hemoglobin. III. A qualitative method for identifying abnormalities of the polypeptide chains of hemoglobin. *Blood,* **24**: 750-756, 1964.
48) Ohba, Y., Miyaji, T., and Shibata, S.: A simple method for detection of chain anomaly of abnormal hemoglobin in hemolysate without preliminary purification. *Acta. Haem. Jap.,* **29**: 128-134, 1966.
49) Itano, H. A., and Robinson, E.: Formation of normal and doubly abnormal hemoglobins by recombination of hemoglobin I with S and C. *Nature,* **183**: 1799-1800, 1959.
50) 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.*,: 675-681, 1962.
51) Iuchi, I., Takeda, I., Miyaji, T., and Shibata, S.: Artificial synthesis and hybridization of Hb M_{Iwate}. *Acta Haem. Jap.*, **27**: 425-430, 1964.
52) Ingram, V. M.: Abnormal human hemoglobins. I. The comparison of normal human and sickle- cell hemoglobins by "Fingerprinting". *Biochim. Biophys. Acta,* **28**: 539-545, 1958.
53) Baglioni, C.: An improved method for the fingerprinting of human hemoglobin. *Biochim. Biophys. Acta,* **48**: 392-396, 1961.
54) Miyaji, T., Ueda, S., Shibata, S., Tamura, A., and Sasaki, H.: Further studies on the fingerprinting of Hb M_{Iwate}. *Acta Haem. Jap.*, **25**· 169-175, 1962.
55) Miyaji, T., and Shibata, S.: unpublished data.
56) Perutz, M. F.: Structure and function of haemoglobin. I. A tentative atomic model of horse oxyhaemoglobin. *J. Mol. Biol.*, **13**: 646-668, 1965.
57) Perutz, M. F., Kendrew, J. C., and Watson, H. C.: Structure and function of haemoglobin. II. Some relations between polypeptide chain configuration and amino acid sequence. *J. Mol. Biol.*, **13**: 669-678, 1965.
58) Suzuki, T., Hayashi, A., Yamamura, Y., Enoki, Y., and Tyuma, I.: Functional abnormality of hemoglobin M_{Osaka}. *Biochem. Biophys. Res. Commun.*, **19**: 691-695, 1965.
59) Suzuki, T., Hayashi, A., Shimizu, A., and Yamamura, Y.: The oxygen equilibrium of hemoglobin $M_{Saskatoon}$. *Biochim. Biophys. Acta.*, **127**: 280-282, 1966.
60) Morimoto, H., and Imai, K.: Unpublished data.
61) Hayashi, A., Shimizu, A., Yamamura, Y., and Watari, H.: Hemoglobin M: Idendification of Iwate, Boston and Saskatoon variants. *Science*, **152**: 207-208, 1966.
62) Hayashi, A., Shimizu, A., Yamamura, Y., and Watari, H.: Electron spin resonance of haemoglobin M_{Iwate} and M_{Osaka}. *Biochim Biophys. Acta,* **102**: 626-628, 1965.
63) Ingram, V. M.: The Hemoglobins in Genetics and Evolution. Colombia University Press (New York, London), 1963.
64) Hayashi, A.: Personal communication.
65) Betke, K.: Hämoglobin M: Typen und ihre Differenzierung (Übersicht). Lehmann, H., and Betke, K.: Haemoglobin Colloquium. Thieme (Stuttgart), 1962 (pp. 39-47).
66) Heller, P., Weinstein, H. G, Yakulis, V. J., and Rosenthal, I. M.: Hemoglobin $M_{Kankakee}$, a new variant of hemoglobin M. *Blood*, **20**: 287-301, 1962.
67) Jaffe, E. R., and Heller, P.: Methemoglobinemia in man. Moore, C. V., and Brown, E. B.: *Progress in Hematology,*Grune & Statton (New York, London), 1964 (pp. 48-71)
68) Jones, R. T., Coleman, R. D., and Heller, P.: The chemical structure of Hgb M_{Iwate} ($M_{Kankakee}$). *Fed. Proc.*, **23**: 173-173, 1964.
69) Shibata, S.: Hemoglobinopathies of the Japanese. *Proc. 16th General Assembly of the Jap. Med. Congr.* (1963), vol. 1, (in Japanese), Nippon Isho Shuppan Kyokai (Tokyo), 1963, (pp. 605-619).
70) Heller, P., Coleman, R. D. and Yakulis, V. J.: *Plenary Sessions of the XIth Congress of*

the International Society of Haematology, Government Printer (Sydney), 1966 (pp. 427-437).
71) Lehmann, H., and Huntsman, R. G.: *Man's Haemoglobins*. North Holland (Amsterdam), 1966.
72) Shibata, S., Miyaji, T., Ohba, Y., and Yamamoto, K.: Unpublished data.
73) Dacie, J. V., and Lewis, S. M.: *Practical Haematology*, Churchill (London), 1963.
74) Maruya, A., Kono, M., Harada, M., Miyazaki, T., Niizaki, R., Nagano, K., Aoyama, H., and Shiraishi, T.: Some family studies of hereditary nigremia. *Acta Haem. Jap.*, **25**: 331-331, 1962.
75) Hansen, H., Jagenburg, R., and Reinand, T.: A new abnormal Fe-hemochromogen as a cause of hereditary cyanosis. *Acta Chem. Scandinav.*, **9**: 1035-1036, 1955.
76) Sahawi, E., Hunger, H., and Betke, K.: Sporadic appearance of Hb M (Boston type ?) in a middle German family. *Schweiz. Med. Wschr.*, **92**: 1090-1112, 1962.
77) Toenz, O., Simon, H. A., and Hasselfeld, W.: Untersuchung einer grossen Hämoglobin-M-Sippe. *Schweiz. Med. Wschr.*, **92**: 1311-1319, 1962.
78) Pick, C. and Tönz, O.: Nature of haemoglobin $M_{Oldenburg}$. *Nature*, **210**: 1182-1182, 1966.
79) Pisciotta, A. V., Ebbe, S. N. E., and Hinz, J. E.: Clinical and laboratory features of two variants of methemoglobin M disease. *J. Lab. & Clin. Med.*, **54**: 73-87, 1959.
80) Overly, W. L., Rosenberg, A., and Harris, J. W.: Hemoglobin $M_{Reserve}$. *J. Lab. & Clin. Med.*, **69**: 62-87, 1967.
81) Murawski, K., Szymanowska, Z., and Kozlowska, J.: A new variant of abnormal methaemoglobin: Hb M_{Radom}. *Biochim. Biophys. Acta*, **69**: 442-444, 1963.
82) Hobolth, N.: Haemoglobin M_{Arhus}. I. Clinical family study. II. Studies on a new haemoglobin which causes congenital methaemoglobinemia. *Acta Paediat. Scandinav.*, **54**: 357-362; 363-368, 1965. Quoted by 84) Farmer et al.
83) Baltzan, D M., and Sugarman, H.: *Canad. Med. Ass. J.*, **62**: 348, 1950. Quoted by 84) Farmer, et al.
84) Farmer, M. B., Lehmann, H., and Raine, D. N.: Two unrelated patients with congenital cyanosis due to hemoglobinopathy M. *Lancet*, Oct. 10: 786-789, 1964.
85) Betke, K., and Kleihauer, E.: *Schweiz. Med. Wschr.*, **92**: 1316, 1962. Quoted by 84) Farmer et al.
86) Josephson, A. M., Weinstein, H. G., Yakulis, B. S., Singer, L., and Heller, P.: A new variant of hemoglobin M disease: Hemoglobin $M_{Chicago}$. *J. Lab. & Clin. Med.*, **59**: 918-925, 1962.
87) Betke, K., Gröschner, E., and Bock, K.: Properties of a further variant of hemoglobin M. *Nature*, **188**: 864-865, 1960.
88) Kiese, M., Kurz, H., und Schneider, C.: Chronische Hämoglobinämie durch pathologischen Blutfarbstoff. *Klin. Wschr.*, **34**: 957-961, 1956.
89) Betke, K., and Kleihauer, E.: Hemoglobin anomalies in the German population. *Schweiz. Med. Wschr.*, **92**: 1316-1318, 1962; Betke, K., Heilmeyer, L., Huisman, T. H. J., and Kleihauer, E.: A new type of hemoglobin M. *IXth International Congress of the International Society of Hematology* (Mexico City, 1962). Abstract.
90) Jones, R. T., Brimhall, B., Huisman, T. H. J., Kleihauer, E. and Betke, K.: Hb Freiburg: Abnormal hemoglobin due to deletion of a single amino acid residue. *Science*, **154**: 1024-1027, 1966.
91) Hollán, Z. R.: Hemoglobins and hemoglobinopathies. *Orv. Hetil.* **105**: 1921, 1964. Quoted by 80) Overly et al.
92) Kerstein, H. G., and Kleihauer, E.: Hemoglobin $M_{Cologne}$, a new hemoglobin variant. Simultancous report on the differential diagnosis of cyanosis. *Med. Welt*, **30**: 1607, 1964. Quoted by 80) Overly et al.
93) Hollán, S. R.: Analysis of a new case of hemoglobin M trait. *IXth Congress of the International Society of Hematology* (Mexico Citx, 1962), Abstract.
94) Lockhart, A., Scebat, L., and Lenegre, J.: A propos de deux observations de cyanose congenital recessive et hemoglobinose M. *Bull. Soc. Med. Hop. Paris*, **113**: 877, 1962. Quoted by 82) Hobolth.

95) Heilmeyer, L.: *Spectrophotometry in Medicine* (Trans. by Jorden A.) Hilger (London), 1943.
96) Hayashi, A., Etiological inquiry into hemoglobinopathy, with special reference to Hb M disease. *J. Clin. Sci.*, **1** : 1-9, 1965. (in Japanese).
97) Hayashi, A., Shimizu, A., Suzuki, T., and Yamamura, Y.: The properties of hemoglobin M. Reactivity of methemoglobin M to cyanide, aziide and fluoride. *Biochim. Biophys. Acta*, **140**: 251-257, 1967.
98) Hayashi, A. Shimizu, A., and Suzuki, T.: Hemoglobin M and its functional abnormality. *Nihon-rinsho*, **25**: 1435-1443, 1967.

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