KAWASAKI MED. J. VOL. 11 : NO. 1, 41-56 (1985)

Relation Between Polycythemia and Function of Hemoglobin with Amino Acid Substitution in $\alpha_1\beta_2$ Contact

Hb Chesapeake [α92 (FG 4) Arg→Leu] and Hb J Cape Town [α92 (FG 4) Arg→Gln]

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Accepted for Publication on February 26, 1985

ABSTRACT. Two variants of abnormal hemoglobins with increased oxygen affinity, Hb Chesapeake (α92 Arg→Leu) and Hb J Cape Town (α92 Arg → Gln), were detected by isoelectric focusing during a population survey of residents of Aichi Prefecture. This was the first time for these abnormal hemoglobins to be found in Japanese.

Following this discovery, a comparative study of the two variants was carried out employing isoelectric focusing, structural analysis (fingerprinting and amino acid analysis), functional analysis (oxygen equilibrium), dissociation into dimers, autoxidation, biosynthesis, and plasma erythropoietin level measurement.

The carriers of Hb Chesapeake, who were heterozygous for the abnormal hemoglobin, showed mild polycythemia. Those of Hb J Cape Town, however, were free of polycythemia, although their hematological values (RBC, Ht and Hb) were close to the upper limits of the normal ranges. The results of the present study support the view that increased production of erythropoietin due to a tissue oxygen deficiency is not the only decisive factor for causation of polycythemia in variant hemoglobins with high oxygen affinity.

Key words : Abnormal hemoglobin — Hb Chesapeake — Hb J Cape Town — Polycythemia — Erythropoietin

To date more than 400 abnormal hemoglobin variants have been recorded worldwide.[1] In Japan, about 100 variants have been detected. Nearly a third of these are associated with clinical symptoms, either major or minor. These symptoms are anemia, cyanosis, and polycythemia.[2] Polycythemia has usually been ascribed to secondary tissue oxygen deficiency and increased erythropoietin production arising from the increased oxygen affinity of such abnormal hemoglobins.[3] These abnormal hemoglobins possess amino acid substitution mainly in one of the following three sites : 1. the $\alpha_1\beta_2$ regions, 2. the 2,3-DPG binding sites, or 3. the region of the β chain N-termini. Of these three, substitution has been observed in the first in a Hb Chesapeake family reported by Charache *et al.*[3,4] in which polycythemia was concomitant with the presence of this abnormal hemoglobin. Hb Chesapeake and Hb J Cape Town[5] were detected for the first time in Japan during a hemoglobinopathy survey we carried out in Aichi Prefecture in 1983. Following this discovery, a comparative study of

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the functions of Hb Chesapeake and Hb J Cape Town was undertaken, since both of these hemoglobins have amino acid substitution at the same site of the α chain, namely at α 92 Arg (FG 4), which is located at the $\alpha_1\beta_2$ interface.[6] This residue is replaced by Leu in Hb Chesapeake and by Gln in Hb J Cape Town. Hb Chesapeake is said to have greater oxygen affinity than Hb J Cape Town and has been reported to cause polycythemia. In Hb J Cape Town carriers so far examined, on the other hand, signs of polycythemia have been absent or extremely slight. The present paper describes the results of our comparative study, with special reference to structure, function, biosynthesis, autoxidation, and dissociation into dimers.

MATERIALS AND METHODS

1) All blood samples for the present investigation were drawn from the antecubital vein and prevented from coagulating by heparin or ethylenediaminetetracetic acid (EDTA-3K).

2) Chemical and hematological laboratory tests were carried out using standard methods.[7–9]

3) 2,3-DPG (2,3-diphosphoglycerate) concentrations of red blood cells were measured according to 2,3-DPG measurement kit (Sigma Laboratory, California) instructions.

4) The hemolysate was prepared by the standard method.[10]

5) Isoelectric focusing of the hemolysate was done at a range of pH 6–9 using of a polyacrylamide gel plate containing Ampholine [1.6% mixture of two types of Ampholine with pH ranges of 7–9 and 3.5–10 (4 : 1 by volume)].[11] Isoelectric focusing was carried out at a constant voltage of 200 V in a cooling chamber (ca. 10°C) for 16 hrs.

6) Hemoglobins were purified by elution of isoelectrofocused hemoglobin bands into distilled water and their concentrations were adjusted to appropriate levels by the vacuum aspiration method using collodion bags.

7) The oxygen affinity of whole blood samples was measured at 37°C by the method of Imai *et al.*[12] after dilution 150 times with isotonic phosphate buffer solution. The solutions of hemoglobins purified by isoelectric focusing were examined for oxygen dissociation curves in 0.05 M Tris buffer solution (pH 7.9) or 0.05 M bis-Tris buffer solution (pH 7.4–7.5 and pH 6.9) containing 0.1 M NaCl at 25°C. The final concentration of hemoglobin was adjusted to 60 μM on heme basis. The effect of organic phosphate on the oxygen affinity of individual hemoglobins was investigated after addition of 2 mM 2,3-DPG or 2 mM IHP (inositol hexaphosphate) to the purified hemoglobin dissolved (final concentration 60 μM on heme basis) in 0.05 M bis-Tris-0.1 M NaCl buffer solution (pH 7.4 or 7.5) at 25°C.

8) The hemoglobin composition of the hemolysate was determined by measuring the absorbance (at 415 nm) of the eluates of the individual hemoglobin bands, which were isoelectrofocused on gel plates and then eluted with 0.1 M sodium-potassium phosphate buffer (pH 7.4) containing KCN (0.01%).

9) The content of Hb F was determined by the alkali denaturation method,[13] and that of Hb A_2 by cellulose acetate membrane electrophoresis.[14]

10) Hemoglobin instability was examined by Carrell's isopropanol precipitation test.[15]

11) Abnormal hemoglobin chains were detected by urea dissociation cellulose acetate membrane electrophoresis.[16]

12) Structural analyses were carried out as follows. Globin was prepared by the method of Anson and Mirsky.[17] Abnormal globin chains were separated on a CM-cellulose (CM-52, Whatman Co.) column with phosphate buffered 8 M urea solution according to the method of Clegg *et al.*[18] with slight modification (column size : 13×250 mm ; 5 mM or 35 mM Na_2HPO_4-8 M urea-50 mM mercaptoethanol, pH 6.8). A linear gradient from 5 mM to 35 mM was made with a two chamber-vessel containing 150 ml of each. Flow rate : 0.3 ml / min ; fraction size : 3.75 ml/tube). Separated abnormal globin chains were dialyzed against water, lyophilized and gel-filtrated on a Sephadex G-25f column using 0.2 M acetic acid to remove the urea-phosphate buffer completely and lyophilized again. The purified abnormal globin chains were digested overnight with TPCK-trypsin (Cooper Biomedical Co.) at room temperature at pH 8.2. Then the digest was subjected to fingerprinting on a cellulose thin layer (Chromagram Sheet, Eastman Kodak Co.).[19] An abnormal peptide spot was cut from the fingerprint map, eluted with 10% acetic acid, lyophilized and hydrolyzed with 6 N HCl at 105°C for 22 hrs. The amino acid composition of the abnormal peptide was analyzed in an automatic amino acid analyzer (Yanaco amino acid analyzer L-7).

13) The rate of spontaneous oxidation by atmospheric oxygen was measured by the method of Rifkind[20] with minor modification. This was done using the purified hemoglobin solutions (concentration : 60 μM on heme basis) in a 0.1 M phosphate buffer (pH 7.4, pH 6.6, at 37°C) with or without 0.1 mM EDTA. Methemoglobin contents were determined spectrophotometrically at 560, 576, and 630 nm (van Asserdelft and Zijlstra[21], 1975) after 0, 2, 4, and 6 hrs incubation.

14) Dissociation equilibrium into dimers was studied using the method of Rosa *et al.*[22] with minor modification. The hemolysate was stored in CO form at 4°C until analysis. carboxy-hemoglobin was converted to oxy-hemoglobin by flash photolysis before use. Solutions of Hb A and abnormal hemoglobin at exactly the same concentration (60 μM on heme basis) were prepared in 0.01 M phosphate buffer, pH 7.00 (containing 0.09 M NaCl and 0.01% KCN). The hemoglobin solutions were fed through a Sephadex G-75 column (1.0×60 cm) from the bottom by a peristaltic pump (flow rate : 0.25 ml/min). The effluent from the top of the column was introduced into a flow cell photometer measuring at 280 nm in a cold room (4°C). The column was first equilibrated with the Hb A solution. The abnormal hemoglobin began to flow successfully to Hb A when the 4-way valve was switched to the abnormal hemoglobin solution. Then the dissociation index was calculated.[34]

15) Erythropoietin was measured by the Hemagglutination Inhibition Test at the Special Reference Laboratories, Inc.

16) Hemoglobin biosynthesis using fresh heparinized peripheral blood was carried out in the following way.[23] Red blood cells collected by washing the peripheral blood (ca. 5 ml) with physiological saline (5 times) were centrifuged at 15,000 rpm for 15 min in a Kubota centrifuge Model KM-15200 in

a cooling chamber at 4°C. A 0.2 ml aliquot of the reticulocyte-enriched erythrocyte top layer was suspended in 1.0 ml of Lingrel-Borsook's amino acid culture medium[24] containing ^{3}H-leucine (100 μCi) and then incubated at 37°C for 2 hrs. After incubation the cells were washed with physiological saline five times to remove the incubation mixture and excess ^{3}H-leucine, after which they were lysed by adding two volumes of 0.1% saponin solution. The lysate was then passed through a millipore filter (pore size : 1.2 μm) to obtain limpid hemolysate containing biosynthesized hemoglobins.

The globin prepared from the hemolysate containing the abnormal hemoglobin together with Hb A was chromatographed on a CM-52 column to separate the globin chains using the method of Clegg *et al.*[18] with slight modification. The smaller size column (0.6 × 10.0 cm) was prepared with CM cellulose (CM-52, Whatman Co.). The procedure for the separation of globin chains was the same as described in 12). The fractionated solutions were measured at 280 nm. Next, one ml of each fraction was poured into a vial containing 6 ml of Scintisol (Scintisol 500, Dojin Laboratories). Then 2 ml of distilled water was added. The fractions and water were mixed by shaking, and the total radioactivities were calculated.[25]

RESULTS

1) *Hematological data of the carriers*

Family X : The propositus (K.H.) was a 32-year-old Japanese male in apparently good health. The only characteristic findings upon physical examination were persistent elevations of the erythrocyte count, hemoglobin, and hematocrit : 5.49 million/μl, 17.2 g/dl, and 49.5%, respectively (Table 1). Blood chemistries were within normal limits.

Families Y_1, Y_2 and Y_3 : Four carriers were found in three families (Y_1, Y_2 and Y_3). Two of them were brothers (Y.K. and H.K., 31 and 29-year-old, males respectively) in family Y_1. The other two carriers (M.I. and M.K., 19 and 58-year-old males, respectively) however, were encountered individually in two different families, Y_2 and Y_3, with no blood relationship. No abnormalities upon physical examination were noted. Hematological data are shown in Table 1.

TAELE 1. Hematological data obtained from Family X and Families Y.

	Age	Sex	RBC $\times 10^4/\mu$l	Hb g/dl	Ht %	MCV fl	MCH pg	MCHC %	Retic %	Abnormal Hb %	Hb F % (0.1–1.1)	Hb A_2 % (2.2–3.2)
Family X (Hb Chesapeake)												
K.H.(X)	32	M	549	17.2	49.5	90	31.3	34.7	1.5	21.8	0.8	2.5
Family Y (Hb J Cape Town)												
Y.K.(Y_1)	31	M	499	15.5	45.4	91	31.1	34.1	0.4	22.3	1.6	2.6
H.K.(Y_1)	29	M	509	16.4	47.2	93	32.2	34.7	0.4	22.0	2.4	2.5
M.I.(Y_2)	19	M	479	14.5	43.6	91	30.3	33.3	0.7	22.0	0.3	3.1
M.K.(Y_3)	58	M	529	16.4	49.0	93	31.0	33.3	0.9	23.0	0.4	2.8
mean			504	15.7	46.3	92	31.2	33.9	0.6	22.3	1.2	2.8

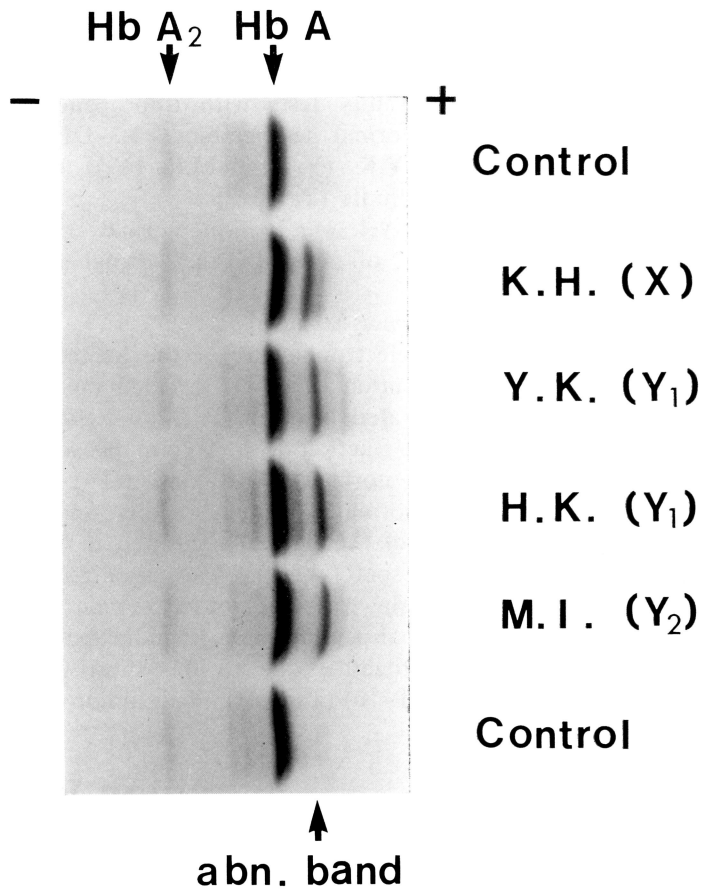

Fig. 1. Isoelectric focusing of the hemolysates on an ampholine-polyacrylamide gel plate (pH range 6-9) K.H. (X) : Hb Chesapeake (Family X) Y.K: (Y_1), H.K. (Y_1), M.I. (Y_2) : Hb J Cape Town (Families Y).

TABLE 2. 2, 3-DPG was measured for Hb Chesapeake and Hb J Cape Town.

	Whole blood μmol/ml (1.6-2.6)	Hb μmol/gr (10.5-15.1)	Packed cell μmol/ml (4.5-5.1)
Hb Chesapeake			
K.H. (X)	2.44	15.0	5.03
Hb J Cape Town			
Y.K. (Y_1)	2.58	16.6	5.68
H.K. (Y_1)	2.41	14.7	5.11
M.I. (Y_2)	1.89	13.1	4.34
M.K. (Y_3)	2.66	16.2	5.42
mean	2.39	15.2	5.14

In all of these carriers, although blood hemoglobin concentrations were within normal limits, they were at the highest range of these limits.

Isoelectric focusing of the hemolysates of the carriers demonstrated abnormal fast-moving bands (Fig. 1). Instability tests with isopropanol were negative with all specimens of purified abnormal hemoglobins. 2,3-DPG contents were elevated slightly in two carriers (Y.K. (Y_1) and M.K. (Y_3)), but in the other carriers they were within normal limits (Table 2).

The plasma erythropoietin level was within normal range, being 38 mIU/ml (control : 28-88) in K.H. of family X and normal also in family Y carriers.

2) *Structural studies of the abnormal hemoglobins*

Cellulose acetate membrane electrophoresis of the hemolysates in 5.5 M urea-Tris-EDTA-borate buffer solution (pH 8.3) revealed the presence of abnormal α chain bands which migrated more anodally than the normal α chain band. The fingerprint of the soluble fraction of the tryptic digest of the abnormal α chains revealed the absence of normal αT-10 and αT-11 peptide spots. However, in the map of the abnormal α^X chain an abnormal spot (abn. X) was seen in the uppermost level of the neutral zone and in the case of the abnormal α^Y chain, an abnormal spot (abn. Y) was visualized just above the location corresponding to the missing αT-11 spot in the neutral zone (Fig. 2). Amino acid analysis of the abnormal peptide (abn. X) indicated that the αT-10 and αT-11 peptides were linked together due to the substitution of Leu for Arg at α92 (the C-terminus of αT-10) (Table 3). This amino acid substitution

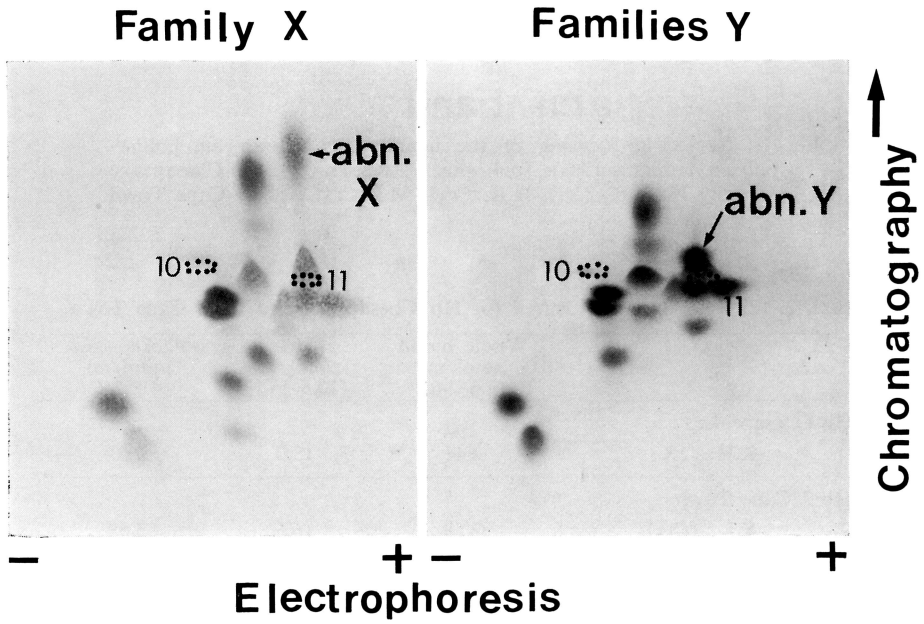

Fig. 2. Fingerprint maps of the soluble fraction of the tryptic digest of the abnormal α chain (Left : Hb Chesapeake (Famly X), Right : Hb J Cape Town (Families Y)). The spots encircled by dotted lines refer to the missing spots. Abn. X and Y iudicate the new peptide spots.

TABLE 3. Amino acid composition of the abnormal peptides from abn. X (Hb Chesapeake) and abn. Y (Hb J Cape Town).

amino acids	abn. X	abn. Y	αT-10-11
Lys	1.06	1.02	1
Arg			1
Asp	2.05	1.99	2
Glu		1.04	0
Pro	1.02	1.08	1
Val	2.06	1.94	2
Leu	1.77	0.96	1
Phe	1.04	0.97	1

Normal : 91 92 93 94 95 96 97 98 99
Leu—Arg—Val—Asp—Pro—Val—Asn—Phe—Lys
|←αT-10→|←——————αT-11——————→|

Arg→Leu at α92 was identical to that of Hb Chesapeake.[3]

Amino acid analysis also showed the abnormal spot (abn. Y) to be a linkage product of αT-10 and αT-11 peptides due to replacement of Arg (α92) by either glutamic acid or glutamine at the C-terminus the αT-10 peptide. The abnormal peptide spots in the neutral zone suggest that the abnormal band of Hb J Cape Town migrated closer to the anode than the normal α chain band by one negative charge difference. Accordingly, it is thought that there was a loss of one positive charge of Arg due to the substitution of Gln for Arg. If Arg were displaced by Glu, there would be a loss of two positive charges. The amino acid substitution in this abnormal hemoglobin is, therefore, α92 Arg→Gln, which is seen in Hb J Cape Town.[5]

These were the first instances of detection of Hb Chesapeake and Hb J Cape Town in Japanese people.

3) *Oxygen equilibrium studies*

The role of α92 Arg is interest because it corresponds to the $\alpha_1\beta_2$ contact. The whole blood of the abnormal hemoglobin samples from the 5 carriers of our study showed high oxygen affinity. The P_{50} (the oxygen pressure under which hemoglobin is half-saturated with oxygen) were 21.0 mmHg at 37°C and pH 7.4 for Hb Chesapeake (Family X) and 23.3 mmHg, and 22.2 mmHg (mean 23.1 mmHg) for Hb J Cape Town (Families Y) in contrast to 27.0 mmHg for the blood sample of a normal subject.

The purified abnormal hemoglobins also showed high oxygen affinity (Table 4, Fig. 3). The P_{50} of Hb Chesapeake was 1.1 mmHg (Hb A = 5.6 mmHg) at pH 7.4 and 25°C. The P_{50} of Hb J Cape Town was 1.7 mmHg (Hb A = 3.8 mmHg) at pH 7.5 and 25°C. The Bohr effect and organic phosphate effect were normal.

4) *Autoxidation*

Figure 4 compares the rates of autoxidation of Hb Chesapeake (Family X), Hb J Cape Town (Families Y), and Hb A, which were isolated and purified by isoelectric focusing. The hemoglobin solutions (60 μM on heme basis) were incubated in 0.1 M phosphate buffer, pH 6.6, at 37°C. In the absence of EDTA, methemoglobin was produced with greater rapidity from Hb Chesapeake,

TABLE 4. Oxygen binding properties of Hb Chesapeake, Hb J Cape Town and Hb A.

	Hb Chesapeake (Family X)		Hb A	
	P_{50}mmHg	P_{50}^{op} / P_{50}^{free}	P_{50}mmHg	P_{50}^{op} / F_{50}^{free}
pH 7.9	0.7		3.8	
pH 7.4	1.1		5.6	
pH 6.9	1.8		8.8	
pH 7.4 (2mM DPG)	2.8	2.7	14.9	2.7
pH 7.4 (2mM IHP)	7.9	7.4	47.4	8.5
Bohr effect (pH 6.9–7.9)	−0.37		−0.42	
Hill's n (pH 7.4)	1.5		2.9	

	Hb J Cape Town (Families Y)		Hb A	
	P_{50}mmHg	P_{50}^{op} / P_{50}^{free}	P_{50}mmHg	P_{50}^{op} / P_{50}^{free}
pH 7.9	1.2		2.6	
pH 7.5	1.7		3.8	
pH 6.9	3.5		7.7	
pH 7.5 (2mM DPG)	4.7	2.8	11.0	2,9
pH 7.5 (2mM IHP)	13.4	7.9	37.7	9.9
Bohr effect (pH 6.9–7.9)	−0.46		−0.47	
Hill's n (pH 7.5)	2.2		2.9	

Experimental conditions : in 0.05 M Tris buffer (pH7.9) or 0.05 M bis-Tris buffer (pH 7.4–7.5) containing 0.1 M Cl^- ; at 25°C ; hemoglobin concentration, 60 μM on heme basis.

Bohr effect ($-\Delta$ log P_{50}/ΔpH).

P_{50}^{op} : with organic phosphate

P_{50}^{free} : without organic phosphate

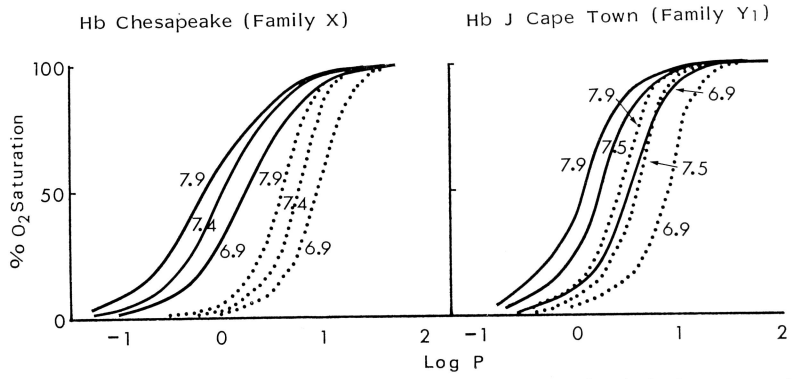

Fig. 3. Left : Oxygen equilibrium curves of Hb Chesapeake (—) in comparison with those of Hb A (···). The lines are written in order from the left, pH 7.9, pH 7.4, pH 6.9. Right : Oxygen equilibrium curves of Hb J Cape Town (—) in comparison with those of Hb A (···). The lines are written in order from the left, pH 7.9, pH 7.5, pH 6.9. The solution had no organic phosphate at 25°C.

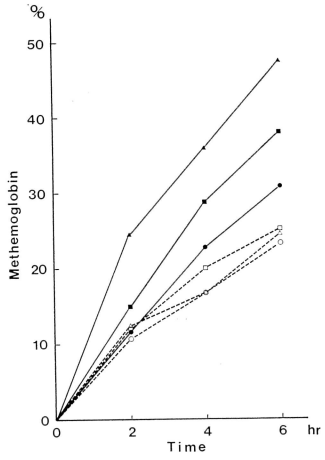

Fig. 4. Rate of autoxidation of purified hemoglobins. Hb A (●), Hb Chesapeake (▲), Hb J Cape Town (■), Hb A + 0.1 mM EDTA (○), Hb Chesapeake + 0.1 mM EDTA (△), Hb J Cape Town + 0.1 mM EDTA (□). Solutions of oxyhemoglobin (60 μM on heme basis) were incubated at 37°C in 0.1 M phosphate buffer of pH 6.6.

TABLE 5. Effect of pH on the autoxidation of Hb A. Hb J Cape Town, and Hb Chesapeake. Specimens containing oxyhemoglobin (60 μM on heme basis) were incubated in 0.1 M phosphate buffer, pH 6.6 and pH 7.4 for 6 hrs at 37°C.

pH	EDT A (0.1 mM)	Methemoglobin (%)		
		Hb A	Hb J Cape Town	Hb Chesapeake
6.6	−	30.8	38.0	47.5
	+	23.3	25.2	24.6
7.4	−	16.1	20.5	20.0
	+	11.5	12.1	13.2

Hb J Cape Town, and Hb A in that order. Addition of 0.1 mM EDTA delayed autoxidation of each hemoglobin. As shown in Table 5, the rate of autoxidation was strongly pH-dependent in the absence as well as presence of EDTA.

5) *Dissociation equilibrium*

The dissociation profiles of tetrameric Hb Chesapeake and Hb J Cape Town are shown in Figure 5. The dissociation of the Hb Chesapeake molecule into dimers decreased with a dissociation index of +4.0, while the Hb J Cape Town molecule underwent accelerated dissociation with a dissociation index of −5.4.

6) *Hemoglobin biosynthesis*

Incorporation of radioactive leucine into globin chains was compared with respect to the total radioactivities (the actual radioactivity count) (Table 6). The ratio of $\alpha^{\text{total}}/\beta^{A}$ was 0.92 in Hb Chesapeake and 0.92 in Hb J Cape Town, where α^{total} refers to the sum of the total radioactivities of the normal and the abnormal α chains, and β^{A} is the total activity of the normal β chain. The α and β chains were synthesized in well-balanced equilibrium. The

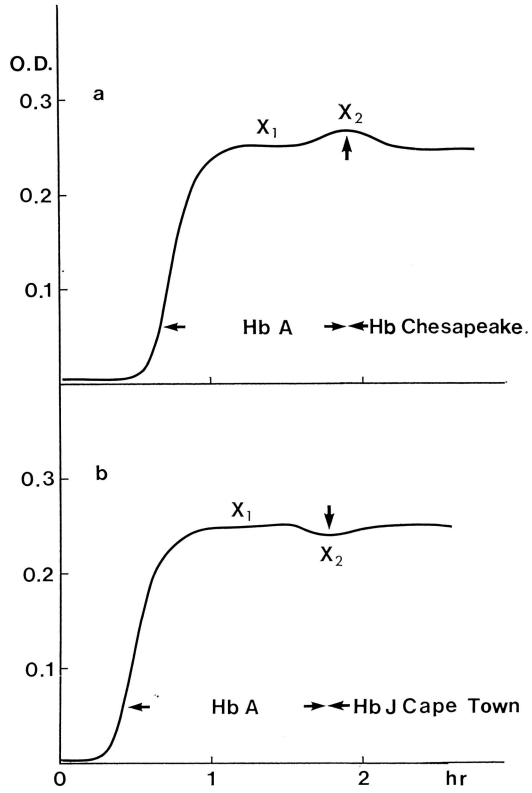

Fig. 5. Dissociation profile of Hb Chesapeake (upper figure) and Hb J Cape Town (lower figure).
a) The upward deflection (X_2 ↑) shows that Hb Chesapeake dissociates less readily than Hb A.
b) The downward deflection (X_2 ↓) suggests that the slowly-eluted dimer of Hb J Cape Town causes the absorbance blank of the foregoing Hb A.
X_1 : base line, X_2 : the deflection peak
dissociation index : $[(X_1-X_2) / X_1] \times 100$

TABLE 6. Incorporation of radioactive leucine into the individual globin chains. The chains were separated by urea-CM cellulose chromatography of the whole hemolysate which was freshly prepared from the reticulocyte-rich red blood cells. The values of incorporation of radioactive leucine into the abnormal α chain of Hb Chesapeake were rectified, taking into consideration the fact that this abnormal chain had one leucine in excess.

Globin	β^A ($\times 10^3$dpm)	α^{abn} ($\times 10^3$dpm)	α^A ($\times 10^3$dpm)	α^{total} / β^A	$\alpha^{abn} / \alpha^{total}$
Hb Chesapeake					
K.H. (X)	52.4	7.2	41.1	0.92	0.15
Hb J Cape Town					
Y.K. (Y_1)	29.2	6.1	20.6	0.91	0.23
H.K. (Y_1)	25.5	6.1	19.4	1.00	0.24
M.I. (Y_2)	38.3	6.9	26.1	0.86	0.21
mean				0.92	0.23

synthesis of the abnormal chains (α^{abn}) occupied 15% of the total α chain synthesis (α^{total}) in Hb Chesapeake ($\alpha^{abn}/\alpha^{total}=0.15$) and 23% in Hb J Cape Town ($\alpha^{abn}/\alpha^{total}=0.23$).

DISCUSSION

It is apparent from the results of structural analysis (fingerprinting and amino acid analysis) described in the preceding paragraph that the two abnormal hemoglobins encountered by us in Aichi Prefecture are identical to Hb Chesapeake and Hb J Cape Town.

Hb Chesapeake was discovered for the first time as a fast-moving hemoglobin by agar and starch gel electrophoresis of hemolysates obtained from an 81-year-old white man of German-Irish extraction by Charache *et al.*[3] in 1966. This discovery was confirmed by starch gel electrophoresis of the same propositus' hemolysate in the same year by Clegg *et al.*[4]

Hb J Cape Town was discovered as a fast-moving hemoglobin by paper electrophoresis of hemolysates obtained from a female of mixed Hottentot and Western-European descent in Cape Town in 1966. Botha *et al.*[5] detected 10 carriers in a family which included 40 members. This fast-moving hemoglobin was similar to Hb Chesapeake in electrophoresis. In 1977, the same hemoglobin was encountered as an abnormal hemoglobin electrophoretically slower than Hb J Baltimore and not different from Hb J Mexico by isoelectric focusing. The hemolysates were of a patient (a 46-year-old man) of French extraction suffering from urticaria. In a family pedigree spanning over four generations eight members had this hemoglobin.

In our Hb Chesapeake family (family X) the propositus was a 32-year-old man and his father had the same abnormal hemoglobin variant. Unfortunately, we were unable to examine other family members. We encountered 3 families of Hb J Cape Town. For convenience sake, they are called family Y_1, family Y_2 and family Y_3, and are collectively called families Y. Two cases (Y.K. (Y_1) : a 31-year-old male, and H.K. (Y_1) : a 29-year-old male) were seen in family Y_1, and one case (M.I. (Y_2) : a 19-year-old male) was encountered in family Y_2. Another case (M.K. (Y_3) : a 58-year-old male) was found in family Y_3. Complete family studies were impossible with families Y.

In our experiment, by means of isoelectric focusing of the hemolysates, Hb J Cape Town migrated a little more closely to the anode than Hb Chesapeake. The main bands of both abnormal hemoglobins accompanied fainter bands of their aged hemoglobins ahead of them, i.e., to the anode side (Fig. 1). The content of Hb Chesapeake in the hemolysate was 21.8% of the total hemoglobin and that of Hb J Cape Town was 21.3% on the average. The content of Hb A_2 in hemolysates was within normal limits (averaging 2.8%) in all the carriers of Hb Chesapeake and Hb J Cape Town. The Hb F content tended to higher in family Y_1, but was normal in Family X.

Another marked difference between Hb Chesapeake and Hb J Cape Town concerns the fingerprints of the abnormal α chains (Hb Chesapeake : α^X, Hb J Cape Town : α^Y). From Figure 2, it is obvious that, in Hb Chesapeake, the abnormal spot (abn. X) is higher in the neutral zone than that of Hb J Cape

Town (abn. Y). Amino acid analysis of the hydrolysate disclosed that both abnormal chains lacked Arg in their compositions, and in its stead Leu and Glu were present in excess in abn. X and abn. Y, respectively. The Glu was ultimately identified with Gln by examination of its negative electric charge by electrophoresis.

Charache (1966) was the first hematologist to investigate the oxygen affinity of Hb Chesapeake. He examined the oxygen equilibrium curve of the abnormal hemoglobin purified from a patient's hemolysate, and noticed that its oxygen affinity was four times as high as that of the hemolysate derived from a normal subject, which was composed almost completely of Hb A, when it was expressed in terms of the ($P_{50}A/P_{50}abn$) ratio.[3] Other authors[26–28] observed the ratio to be 6.1–8.1 and we found it to be 5.2 (Table 7). Hill's constant was n=2.2–1.5 with the exception of that by Charache who did not mention his result. Our own result showed that Hill's constant was n=1.5. In other words, the heme-heme interaction of Hb Chesapeake had distinctly decreased. However, the Bohr effect was within normal limits (Table 4).

TABLE 7. Comparison of the oxygen affinity of Hb Chesapeake with that of Hb J Cape Town.

	Hb Chesapeake				
	Charache *et al.*[3]	Nagel *et al.*[26]	Imai *et al.*[27]	Matsukawa *et al.*[28]	author *et al.*
P_{50}^A/P_{50}^{abn}	4.0	8.0	6.1	8.1	5.2
Hill's n	1.1	1.4	1.2	1.5	1.5
	(Hb A=2.0)	(2.8)	(2.8)	(2.6)	(2.9)
Bohr effect	normal	normal	—	—	normal
Condition					
pH	7.4	7.04	7.4	7.0	7.4
Temp.	10°C	10°C	25°C	25°C	25°C
Buffer	0.1M P.B.	0.1M P.B.	0.05M B.T.		0.05M B.T.
	Hb A= Whole hemolysate of the patient				

	Hb J Cape Town				
	Lines *et al.*[30]	Nagel *et al.*[26]	Gacon *et al.*[29]	Matsukawa *et al.*[28]	author *et al.*
P_{50}^A/P_{50}^{abn}	1.1	2.6	2.0	3.0	2.2
Hill's n	1.5	2.2	—	2.5	2.2
	(2.9)	(2.8)		(2.6)	(2.9)
Bohr effect	normal	normal	—	—	normal
Condition					
PH	7.03	7.04	7.1	7.0	7.5
Temp.	30°C	10°C	25°C	25°C	25°C
Buffer	P.B.		0.05M B.T.		0.05M B.T.
	Hb Abn=Whole hemolysate of the patient				

P.B.=Phosphate Buffer B.T.=bis-Tris Buffer

As to Hb J Cape Town, Lines and McIntosh examined its oxygen equilibrium curve in 1967 with whole hemolysate which contained the abnormal hemoglobin together with Hb A and noticed its increased oxygen affinity (Hb J Cape Town+Hb A : $P_{50}=6.46$, Hb A : $P_{50}=8.91$). The ($P_{50}A/P_{50}abn$) ratio was obtained by Nagel *et al.*[26] Gacon *et al.*[29] and Matsukawa *et al.*[28] with a purified specimen of Hb J Cape Town within a range of from 2.0 to 3.0. In our experiment the ($P_{50}A/P_{50}abn$) ratio was 2.2. It is therefore germane to conclude that Hb J Cape Town is certainly an abnormal hemoglobin of high oxygen affinity, although elevation of oxygen affinity is not so marked in Hb J Cape Town as in Hb Chesapeake. Hb Chesapeake has oxygen affinity 2–3 times higher than that of Hb J Cape Town.

Jones *et al.*[31,32] who discovered Hb Yakima (β 99 Asp→His), an abnormal hemoglobin with increased oxygen affinity, in 1967, suggested that the tetrameric molecule of abnormal hemoglobins of increased oxygen affinity would be less prone to dissociation into two dimers than that of abnormal hemoglobins of decreased oxygen affinity. Bunn[33] tested and corroborated Jones' view with Hb Chesapeake. However, a similar examination has not yet been done with Hb J Cape Town. The result of our observation on the dissociation of Hb Chesapeake and Hb J Cape Town is presented diagrammatically in Figure 4. The dissociation index[34] of Hb Chesapeake was +4.0, while in Hb J Cape Town it was −5.4, indicating that the former is less dissociable than the latter. Accordingly, it is expected that Hb Chesapeake will show greater affinity to oxygen than Hb J Cape Town does.

Autoxidation is apt to occur in acid pH medium. Hb Chesapeake and Hb J Cape Town were examined for their autoxidation in media of pH 6.6 and 7.4 (37°C). The results showed that Hb Chesapeake was autoxidized more easily than Hb J Cape Town (Fig. 5, Table 5). However, no difference was noticed between the two when they were compared with each other at neutral or physiological pH (pH 7.4, at 37°C). When EDTA was added to the medium, autoxidation was distinctly suppressed at pH 6.6 and 7.4, resulting in the disappearance of different autoxidation in both abnormal hemoglobins. Removal of the autoxidation-catalyzing Cu^{++} ion by EDTA is thought to be responsible for this phenomenon.[20] However, it is relevant to suppose that these hemoglobins will undergo autoxidation *in vivo* because the internal milieu of the body is physiologically normal at pH 7.4.

Suspension of deoxy-conformation of the hemoglobin molecule by 2,3-DPG results in a decrease, in the oxygen affinity of hemoglobin, facilitating the release of oxygen from it to the tissue. Reports on the oxygen dissociation curve of erythrocyte suspension have not as yet been met in the literature. The 2,3-DPG content of the whole blood of as judged the Hb Chesapeake carrier was within normal limits by our examination (Table 2), but among the four Hb J Cape Town carriers, the blood samples of two carriers (Y.K. (Y_1), M.K. (Y_3)) were slightly over the upper limit of the normal range. This may contribute to the suppression of the oxygen affinity of hemoglobin in these carriers.

Hb Chesapeake or Hb J Cape Town occupies as much as 20% of the hemoglobins of the whole hemolysate. The high oxygen affinity hemoglobin content of blood of this order is enough to cause a certain degree of deficit in the oxygen supply to tissues. According to Charache *et al.*, the whole blood

sample of his Hb Chesapeake patient was equivalent to cord blood in its increase in oxygen affinity over that in normal adult blood. However, the oxygen affinity of Hb J Cape Town is not thought to be elevated to this level. Theoretically, as has been stated by Adamson, Parer and Stomatoyannopoulos,[35] the tissue oxygen deficiency thus arising should act to cause polycythemia since a deficient supply of oxygen to tissues may be expected to stimulate the production of erythropoietin. Charache, however, could not verify the validity of this theory by his observations of the serum erythropoietin level of his Hb Chesapeake carrier. It did not increase, being 0.03 U per ml.[3] According to Jones *et al.* there was an increase in the daily urinary erythropoietin output in Hb Yakima carriers associated with polycythemia.[32] However, even people who live at high altitudes and are actually polycythemic may occasionally be normal in their serum erythropoietin level. Thus, patients with secondary polycythemia do not always show significant indications of increased production of erythropoietin during their clinical course.

The plasma erythropoietin levels were normal in our family X (Hb Chesapeake) and family Y_1 (Hb J Cape Town). In this respect, it is especially worthwhile to mention Yamaoka's report on the Hirose family. According to him Hb Hirose (β37 Try→Ser) had distinctly high oxygen affinity, (4.2 times as high as that of Hb A) and it constituted about 40% of the total hemoglobins of the carriers of this abnormal hemoglobin, but they were free of polycythemia. He stated that there are various factors responsible for polycythemia in addition to increased production of erythropoietin in the patient of secondary polycythemia. It is therefore thought that, apart from our Hb Chesapeake family, our Hb J Cape Town family is an instance comparable to Yamaoka's Hb Hirose family.

Further study will be required before polycythemia seen in conjunction with abnormal hemoglobin of high oxygen affinity can be ascribed exclusively to the overproduction of erythropoietin due to tissue oxygen deficiency. The tetrameric molecule of hemoglobins of increased oxygen affinity is resistant to dimerization, and this may also contribute to the induction of polycythemia by an unknown machanism. The extent to which the erythrocyte 2,3-DPG is produced (in families Y_1, Y_3 of Hb J Cape Town, for instance) may to a certain degree be connected with polycythemia.

The total α chain, namely the sums of the normal α chain (α^A) and the abnormal α chain (α^X or α^Y), were equally (1 : 1) balanced against the normal β chain (β^A) in production in our family X (Hb Chesapeake) as well as in the families Y (Hb J Cape Town). The balance of α and non-α chain production has been until now thought to be unrelated to the causation of polycythemia.

Acknowledgment

The author wishes to express his deep thanks to Professor Satoshi Ueda, Department of Clinical Pathology, Kawasaki Medical School as well as to President Susumu Sibata, Associate Professor Teruo Harano, Dr. Keiko Harano and Professor Yoshihito Yawata, Kawasaki Medical School, for their kind directions and encouragement throughout the course of this work and to Dr. Kiyohiro Imai, Osaka University, School of Medicine, for his technical guidance in the measurement of oxygen equilibrium curves.

REFERENCES

1) Wrightstone, R.N. : List of abnormal hemoglobins in the world. Augusta, Georsia, U.S.A., International Hemoglobin Information Center (IHIC)-comprehensive Sickle Cell Center. 1983
2) Shibata, S., Miyaji, T. and Ohba, Y. : Abnormal hemoglobin in Japan. Hemoglobin 4 (3-4, special issue) : 395-408, 1980
3) Charache, S., Weatherall, D.J. and Clegg, J.B. : Polycythemia associated with a hemoglobinopathy. J. Clin. Invest. **45** : 813-822, 1966
4) Clegg, J.B., Naughton, M.A. and Weatherall, D.J. : Abnormal human haemoglobins separation and characterization of the α and β chains by chromatography, and the determination of two new variants Hb Chesapeake and Hb J Bangkok. J. Mol. Biol. **19** : 91-108, 1966
5) Botha, M.C., Beale, D., Isaacs, W.A. and Lehmann, H. : Haemoglobin J Cape Town-α_2 92 Arginine→Glutamine β_2. Nature **212** : 792-795, 1966
6) Perutz, M.F., Muirhead, H., Cox, J.M. and Goaman, L.C.G. : Three-dimensional Fourier synthesis of horse oxyhaemoglobin at 2.8 A resolution : The atomic model. Nature **219** : 131-139, 1968
7) Shibata, S., Ueda, S. and Matsuda, N. : Diagnosis based on blood chemical analysis. Tokyo, Igakushoin. 1984 (in Japanese)
8) Dacie, J.V. and Lewis, S.M. : Practical haematology. Edinburgh, London and New York, Churchill Livingstone. 1975
9) Wintrobe, M.M. : Clinical hematology, 8th ed. Philadelphia, Lea and Febiger. 1981
10) Jonxis, J.H.P. and Huisman, T.H.J. : A laboratory manual hemoglobins. Oxford and Edinburgh, Blackwell. 1968
11) Harano, T., Harano, K., Koide, T., Okada, M., Ueda, S. and Shibata, S. : Mass screening method of abnormal hemoglobin. Jap. J. Clin. Path. **28** : 149-152, 1980 (in Japanese)
12) Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirota, W. and Kuroda, M. : Studies on the function of abnormal hemoglobin. I. An improved method for automatic measurement of the oxygen equilibrium curve of hemoglobin. Biochim. Biophys. Acta **200** : 189-196, 1970
13) Betke, K., Marti, H.R. and Schlicht, I. : Estimation of small percentages of foetal haemoglobin. Nature **184** : 1877-1878, 1959
14) Ueda, S., Shibata, S., Miyaji, T. and Ohba, Y. : Routine Hb A_2 estimation by cellulose acetate membrane electrophoresis. Kawasaki Med. J. **1** : 113—120, 1975
15) Carrel, R.W. and Kay, R. : A simple method for detection of unstable haemoglobins. Br. J. Haematol. **23** : 615-619, 1972
16) Ueda, S. and Schneider, R.G. : Rapid differentiation of polypeptide chains of hemoglobin by cellulose acetate electrophoresis of hemolysate. Blood **34** : 230-235, 1969
17) Anson, M.L. and Mirsky, A.E. : Protein coagulation and its reversal. The separation of insoluble globin, soluble globin and heme. J. Gen. Physiol. **13** : 469-476, 1930
18) Clegg, J.B., Naughton, M.A. and Weatherall, J.D. : Abnormal human haemoglobins, separation and characterization of the α and β chains by chromatography, and determination of two new variants, Hb Chesapeake and Hb J Bangkok. J. Mol. Biol. **19** : 91-108, 1966
19) Harano, K., Harano, T., Ueda, S. and Shibata. S. : Mapping and amino acid analysis of tryptic peptides of globin by use of cellulose thin layer. Kawasaki Med. J. **4** : 323-326, 1978
20) Rifkind, J.M. : Copper and the autoxidation of hemoglobin. Biochemistry **13** : 2475-2481, 1974
21) van Assendelft, O.W. and Zijlstra, W.G. : Extinction coefficients for use in equations for the spectrophotometric analysis of haemoglobin mixtures. Anal. Biochem. **69** : 43-48, 1975
22) Rosa, D.C.J., Belkhodja, O. and Bierme. P. : Hemoglobin Toulouse $\alpha_2\beta_2^{66}$ (E10) Lys→ Glu, structure and consequences in molecular pathology. Biochim. Biophys. Acta **236** : 201-207, 1971
23) Harano, T., Ueda, S., Harano, K. and Shibata, S. : Improved method for quantitation of biosynthesized human globin chains in reticulocytes by use of urea cellulose acetate membrane electrophoresis. Proc. Jap. Acad. **56B** : 230-234, 1980
24) Lingrel, J.B. and Borsook, H. : A comparison of amino acid incorporation into the hemoglobin and ribosomes of marrow erythroid cells and circulating reticulocytes of several anemic rabbits. Biochemistry **2** : 309-314, 1963

25) Huisman, T.H.J. and Jonxis, H.J.P. : The Hemoglobinopathies. Techniques of identification. New York and Basel, Marcel Dekker, Inc. 1977
26) Nagel, R.L., Gibson, Q.H. and Jenkins, T. : Ligand binding in Hemoglobin J Capetown. J. Mol. Biol. **58** : 643-650, 1971
27) Imai, K. : Hemoglobin Chesapeake (92α, Arginine→Leucine). J. Biol. Chem. **249** : 7607-7612, 1974
28) Matsukawa, S., Mawatari, K., Yoneyama, Y. and Shimokawa, Y. : Close correlation between Monod-Wyman-Changeux parameters, L and c, and its implication for the stereochemical mechanism of haemoglobin allostery. J. Mol. Biol. **150** : 615-621, 1981
29) Gacon, G., Amegnizin, K. E., Belkhodia, O., Denis, P., Krishnamoorthy, R., Labie, D., Lefrancois, R., Michel, Y., Pasquis, P. and Wajcman, H. : Un Nouveau can d'hemoglobin J Capetown α92 (FG 4) Arg→Gln. Nouv. Rev. Fr. Hematol. **18** : 35-44, 1977
30) Lines, J.G. and McIntosh, R. : Oxygen binding by Haemoglobin J Cape Town ($\alpha_2$92 Arg→Gln). Nature **215** : 297-298, 1967
31) Jones, R.T., Osgood, E.E., Brimball, B. and Koler, R.D. : Hemoglobin Yakima : I. Clinical and Biochemical Studies. J. Clin. Invest. **46** : 1840-1847, 1967
32) Novy, M.J. Edwards, M.J. and Metcalfe, J. : Hemoglobin Yakima : II. High blood oxygen affinity associated with compensatory erythrocytosis and normal hemo dynamics. J. Clin. Invest. **46** : 1848-1854, 1967
33) Bunn, H.F. : Dissociation of Haemoglobin Chesapeake into subunits. Nature **227** : 839-840, 1970
34) Hattori, Y. Ohba, Y. and Miyaji, T. : A sensitive method for detecting subunit dissociation of abnormal hemoglobin. Bull. Yamaguchi Med. Sch. **30** : 11-19, 1983
35) Adamson, J.W., Parer, J.T. and Stamatoyannopoulos, G. : Erythrocytosis associated with hemoglobin Rainier : Oxygen equilibria and marrow regulation. J. Clin. Invest. **48** : 1376-1386, 1969