Oxygen Equilibrium Characteristics of Abnormal Hemoglobins: Hirose ($\alpha_2\beta_2^{37Ser}$), L Ferrara ($\alpha_2^{47Gly}\beta_2$), Broussais ($\alpha_2^{90Asn}\beta_2$), and Dhofar ($\alpha_2\beta_2^{58Arg}$)

SHIGERU FUJITA

From the First Department of Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan

ABSTRACT The oxygen equilibrium characteristics of four structural variants of hemoglobin A were correlated with their amino acid substitutions.

Hemoglobin Dhofar, in which the proline at E2(58)β is replaced by arginine, had normal oxygen equilibrium characteristics.

Hemoglobin L Ferrara, in which the aspartic acid at CD5(47)α is replaced by glycine, and hemoglobin Broussais, in which the lysine at FG2(90)α is replaced by asparagine, both showed a slightly elevated oxygen affinity; nevertheless both demonstrated a normal heme-heme interaction and a normal Bohr effect.

Hemoglobin Hirose, in which the tryptophan at C3(37)β is replaced by serine, showed abnormalities of all oxygen equilibrium characteristics; i.e., increased oxygen affinity, diminished heme-heme interaction, and reduced Bohr effect.

These results suggest that aspartic acid at CD5(47)α and lysine at FG2(90)α are involved in the function of the hemoglobin molecule, despite the fact that these positions are not located directly in the heme or the α-β-contact regions.

Tryptophan at C3(37)β is located at contact between α_1- and β_2-subunits. It is suggested that the substitution by serine might disturb the quarternary structure of the mutant hemoglobin molecule during transition from oxy-form to deoxy-form resulting in an alteration of the heme function.

This work was presented in part at the 33rd Annual Meeting of the Japan Hematological Society.

Dr. Fujita's present address is Division of Human Genetics, Department of Medicine, Cornell University Medical College, New York.

Received for publication 6 August 1971 and in revised form 13 June 1972.

INTRODUCTION

Since Pauling, Itano, Singer, and Wells (1) demonstrated sickle cell hemoglobin in 1949, over a hundred structural variants of human hemoglobin have been reported. In some cases, a substitution of a single amino acid residue alters the functional properties of hemoglobin and the mutant hemoglobin is associated with clinical manifestations.

The introduction of X-ray crystallography analysis facilitated the description of the detailed architecture and the construction of atomic model of hemoglobin molecule (2–6).

Correlated studies of structure and function of hemoglobins have contributed not only to our understanding of the disordered mechanisms resulting from molecular alterations, but also to knowledge of the interrelation between structure and function of the normal hemoglobin molecule (7).

Since 1957, the Biochemical Laboratory of the First Department of Medicine, Faculty of Medicine, Kyushu University has examined electrophoretically over 50,000 blood specimens from successive clinic patients with the aim of detecting hemoglobin variants. As a consequence, 11 kindreds with inherited structural variants have been discovered and in 9 of them the amino acid sequences were identified (8–15). The present paper deals with oxygen equilibrium characteristics of four of these—hemoglobin L Ferrara ($\alpha_2^{47Gly}\beta_2$) (8, 16), hemoglobin Broussais ($\alpha_2^{90Asn}\beta_2$) (13, 17), hemoglobin Dhofar ($\alpha_2\beta_2^{58Arg}$) (12, 18), and hemoglobin Hirose ($\alpha_2\beta_2^{37Ser}$) (14). The functional properties of these hemoglobins were discussed on the molecular level based on the atomic model proposed by Perutz and his colleagues, and comments were made on the pathophysiological mechanisms involved in the functional aberrations.

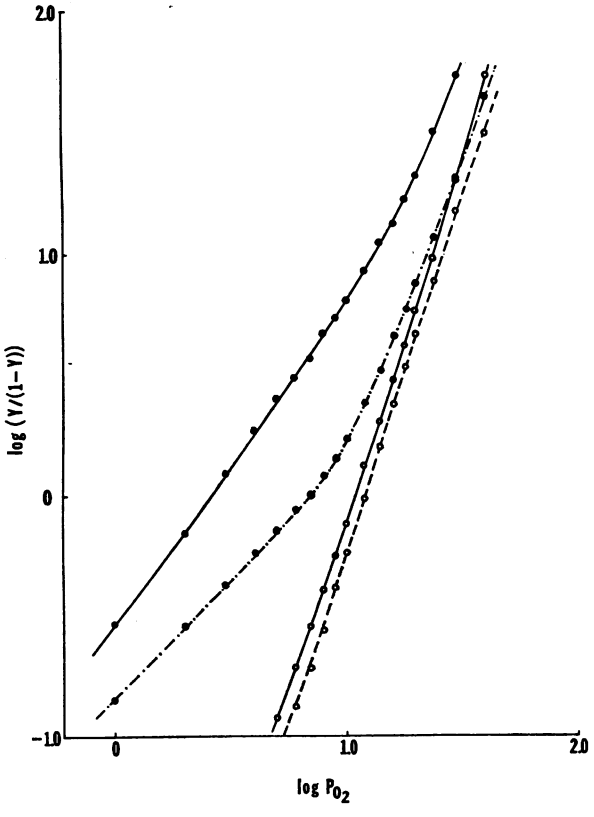

FIGURE 1 Oxygen equilibria of unfractionated hemolysates from a normal adult (O----O) and from a heterozygote for hemoglobin Hirose (●-·-·-●), and those of hemoglobins A (O——O) and Hirose (●——●) isolated from the same hemolysate. The data were obtained at pH 7.01, 20°C. Hemoglobin concentrations were 0.1% in 0.1 M phosphate buffer. The curves were plotted according to Hill's equation.

METHODS

Preparation of hemoglobin components. Blood samples were taken from the cubital veins of normal and affected individuals using heparin sodium as the anticoagulant. The red blood cells were separated from sera and washed four times with cold physiological saline solution. Hemolysates were prepared according to the method of Drabkin (19). Erythrocytes were lysed by mixing 1 vol of washed packed cells, 1 vol of cold distilled water, and 0.5 vol of toluene. From the hemolysates which had been refrigerated overnight, the hemoglobin layer was separated by centrifugation.

Purification of hemoglobin A and abnormal hemoglobins was carried out on starch block electrophoresis in 0.05 M sodium-barbital buffer at pH 8.6 according to Kunkel and Wallenius (20), or on column chromatography of DEAE Sephadex A-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) using Tris-HCl buffer according to Huisman and Dozy (21). The purity of the separated hemoglobins was confirmed by thin starch gel electrophoresis with Tris-EDTA-borate buffer at pH 8.6 (10).

The purified hemoglobins, the unfractionated hemolysates containing abnormal hemoglobins (abnormal hemolysates), and the unfractionated hemolysates from normal individuals (normal hemolysates) were dialyzed against 0.1 M phosphate buffer (Na_2HPO_4–KH_2PO_4) at 4°C for 20 hr. After dialysis, each sample was diluted to a 0.1% hemoglobin concentration and the oxygen equilibrium was measured at 20°C at different pHs. The hemoglobin concentration was determined spectrophotometrically after conversion to pyridine hemochromogen (22).

Measurement of oxygen equilibrium of hemoglobins. The oxygen equilibrium of hemoglobin was recorded automatically as a successive deoxygenation curve according to the method of Imai, Morimoto, Kotani, Watari, Hirata, and Kuroda (23). The oxygen partial pressure (P_{O_2}) in the sample was measured with a Beckman polarographic oxygen sensor, model 39065 (Beckman Instruments, Inc., Fullerton, Calif.), and the percentage of oxyhemoglobin was estimated spectrophotometrically using a monochromatic light beam at 560 mμ. The values were recorded continuously on an X-Y recording chart. The temperature of the samples was measured by a thermistor and maintained at 20°C to within ±0.1°C by thermodules throughout the measurement of oxygen equilibria. The curves were readily reproducible; the maximum standard error was approximately 3% near the half saturation point.

Before and after the oxygen equilibrium measurement, the visible absorption spectra of samples were recorded by

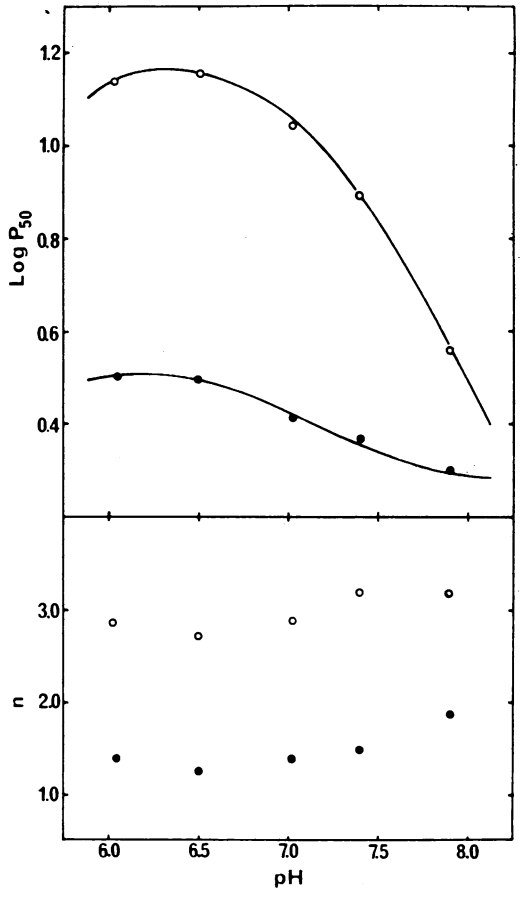

FIGURE 2 pH dependence of oxygen affinity and values of Hill constant n for hemoglobin A (O) and hemoglobin Hirose (●).

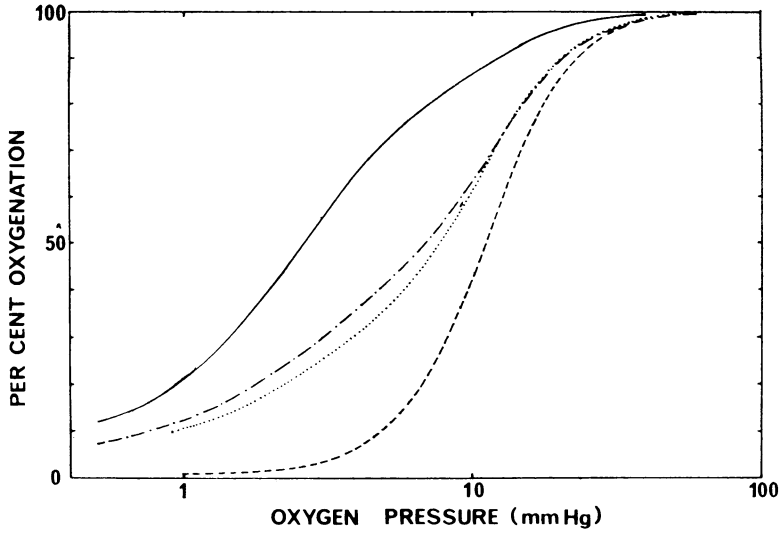

FIGURE 3 Oxygen equilibrium curves of hemolysate from a heterozygote for hemoglobin Hirose (-·-·-·), purified hemoglobin A (----) and purified hemoglobin Hirose (——) under the same conditions as in Fig. 1. The dotted line is the curve calculated from the data of hemoglobin A and hemoglobin Hirose on the assumption that the hemolysate contains 40% hemoglobin Hirose.

a Beckman DK-2 self-recording spectrophotometer (Beckman Instruments, Inc.) in order to assess the amount of methemoglobin which was formed during the measurement of oxygen equilibrium. Methemoglobin in the samples was calculated from extinction coefficients (24). The quantity of methemoglobin formed during the oxygen equilibrium study was less than 7%.

The fractional oxygen saturation of hemoglobin, Y, was calculated by following formula:

$$Y = (OD_{deoxy} - OD) / (OD_{deoxy} - OD_{oxy})$$

where OD_{deoxy} and OD_{oxy} are the optical densities of deoxygenated and oxygenated hemoglobin respectively and OD are the optical densities converted from the transmittances measured during the oxygen equilibrium study.

The oxygen affinity of hemoglobin was expressed by P_{50} which is the oxygen partial pressure at half saturation of hemoglobin with oxygen. pH depndence of oxygen affinity of hemoglobin, i.e. the Bohr effect (25), was estimated from the values of P_{50} at pH values ranging from 7 to 8. The formula $\gamma = \Delta \log P_{50}/\Delta pH$ was used.

Values of n in Hill's equation (26), which is the numerical expression for heme-heme interaction, were calculated from the most linear part of the slope in curves of the $\log(Y/(1-Y))$ plotted against $\log P_{O_2}$.

The oxygen equilibrium studies were carried out within 7 days after the blood had been taken.

RESULTS

Oxygen equilibrium characteristics of hemoglobin Hirose ($\alpha_2\beta_2^{97Ser}$). The oxygen equilibrium curves of purified hemoglobin Hirose and hemoglobin A are shown in Fig. 1. It can be noted that the oxygen equilibrium curve of hemoglobin Hirose is shifted markedly to the left of hemoglobin A and that the slope of the curve is decreased. These findings indicate a increased oxygen affinity and reduced heme-heme interaction of hemoglobin Hirose. The P_{50} at pH 7.01, 20°C of hemoglobin Hirose was only 2.6 mm Hg compared with 11 mm Hg for hemoglobin A. The average value of n for hemoglobin Hirose was 1.48±0.24 (SD, N = 5) compared with 2.97± 0.21 (SD, N = 5) for purified hemoglobin A (Fig. 2). A pH increase from 6.5 to 7.9 raises the n value of hemoglobin Hirose from 1.26 to 1.88. The Bohr effect of hemoglobin Hirose is markedly decreased: the γ-value was -0.13 for hemoglobin Hirose and -0.53 for hemoglobin A.

The unfractionated hemolysate of an individual heterozygous for hemoglobin Hirose contained approximately 40% hemoglobin Hirose. The oxygen equilibrium curve of the abnormal unfractionated hemolysate is shifted to a position approximately intermediate between those of purified hemoglobin Hirose and purified hemoglobin A (Fig. 1). The curve shows a biphasic configuration, more analogous to that of hemoglobin Hirose at the lower part and more analogous to that of hemoglobin A at the upper part of the curve. The oxygen dissociation curves shown in Fig. 3 illustrate the close agreement between the observed dissociation curve in a hemolysate from a heterozygous carrier and the expected curve calculated from the purified mutant and normal hemoglobins.

Oxygen equilibrium characteristics of hemoglobin L Ferrara ($\alpha_2^{47Gly}\beta_2$). As shown in Fig. 4, unfractionated hemolysate which contained approximately 16% hemo-

globin L Ferrara, showed normal functional properties when compared with an unfractionated normal hemolysate.

The oxygen affinity of purified hemoglobin L Ferrara was increased when compared with purified hemoglobin A (Fig. 4). P_{50} at pH 7.02, 20°C was 7.5 mm Hg for hemoglobin L Ferrara and 10.9 mm Hg for hemoglobin A. The average n value in Hill's equation was 2.67±0.24 (SD, N =5) for hemoglobin L Ferrara and 2.91±0.02 (SD, N = 5) for hemoglobin A. The Bohr effect was similar in both hemoglobins (Fig. 5). The γ-value was −0.51 for hemoglobin L Ferrara and −0.56 for hemoglobin A.

Oxygen equilibrium characteristics of hemoglobin Broussais ($\alpha_2^{90Asn}\beta_2$). Unfractionated hemolysate from an individual heterozygous for hemoglobin Broussais showed oxygen equilibrium characteristics similar to that of unfractionated hemolysate from a normal individual (Fig. 6). The abnormal hemolysate contained, however, only 17% hemoglobin Broussais.

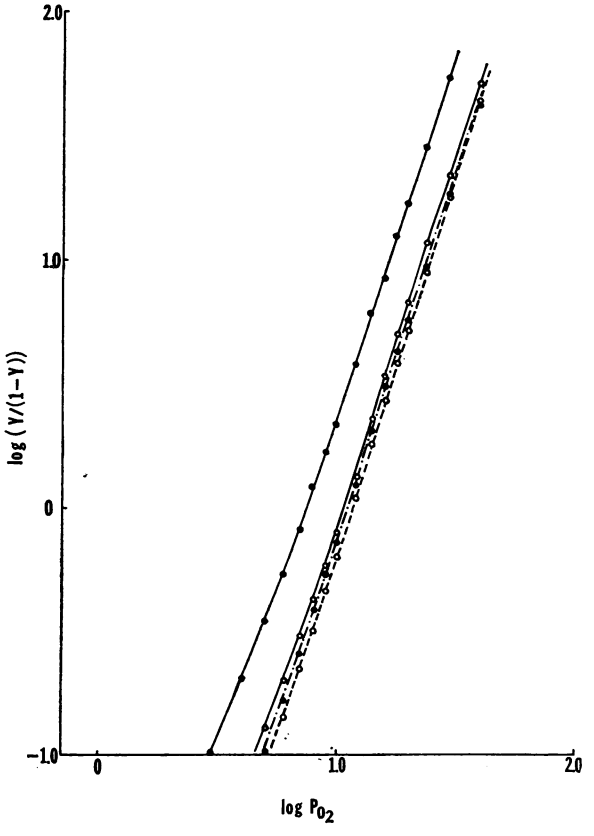

FIGURE 4 Oxygen equilibria of unfractionated hemolysates from a normal adult (○----○) and from a heterozygote for hemoglobin L Ferrara (●-·-·-·●), and those of hemoglobins A (○——○) and L Ferrara (●——●) isolated from the same hemolysate. The data were obtained at pH 7.02, 20°C. Hemoglobin concentrations were 0.1% in 0.1 M phosphate buffer. The curves were plotted according to Hill's equation.

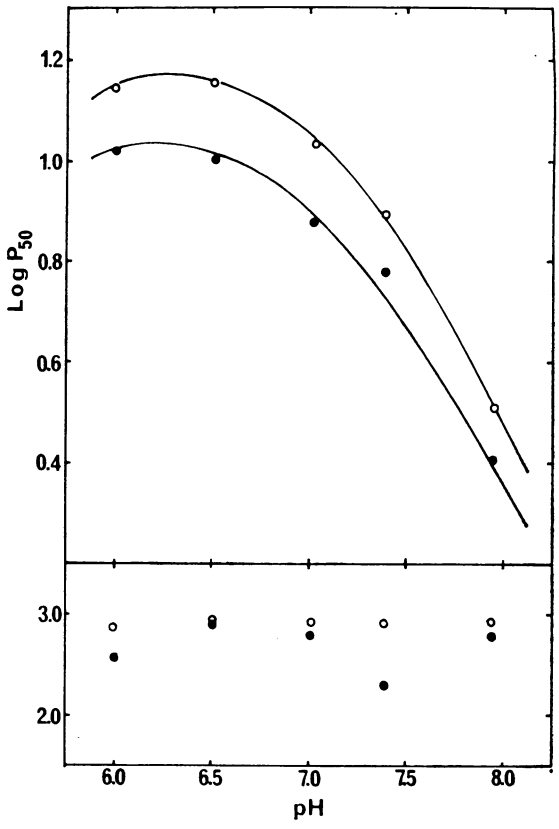

FIGURE 5 pH dependence of oxygen affinity and values of Hill constant n for hemoglobin A (○) and hemoglobin L Ferrara (●).

Oxygen equilibrium curves of purified hemoglobin Broussais and purified hemoglobin A are shown in Fig. 6. The curve for hemoglobin Broussais is shifted slightly left as compared with that of hemoglobin A under the same experimental contditions. P_{50} at pH 7.04, 20°C was 9.6 mm Hg for hemoglobin Broussais and 10.6 mm Hg for hemoglobin A. The average n value in Hill's equation was 2.41±0.16 (SD, N = 5) for hemoglobin Broussais and 2.56±0.13 (SD, N = 5) for hemoglobin A. The Bohr effect was similar in both hemoglobins (Fig. 7).

The oxygen affinity of purified hemoglobin A was slightly higher than that of the unfractionated normal hemolysate. This may imply that the functional properties of the hemoglobin are artificially modified during purification. If the purification procedure affected hemoglobin Broussais to a greater degree than hemoglobin A, it might result in the slightly increased oxygen affinity in the former. Before and after the measurement of oxygen equilibrium, the visible spectrum (450–700 mμ) of purified hemoglobin Broussais was compared with purified hemoglobin A. No differences were discovered.

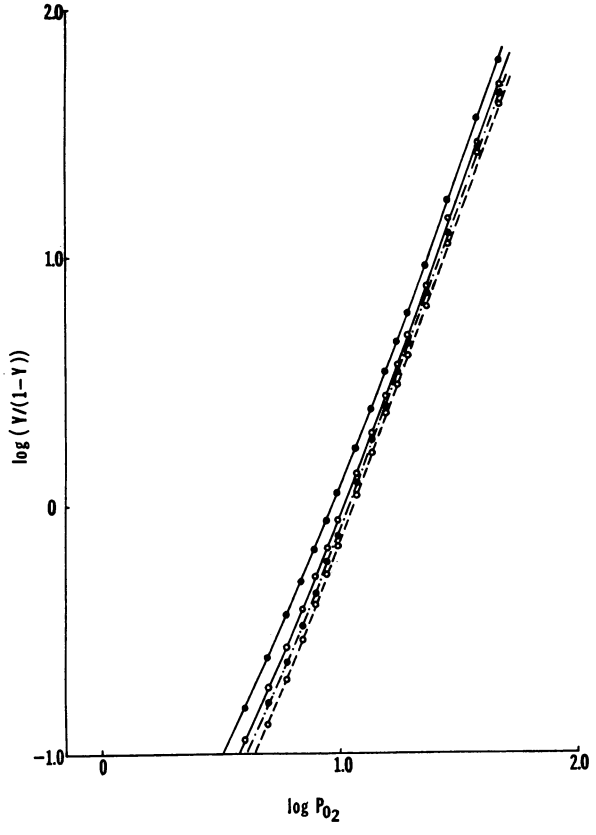

FIGURE 6 Oxygen equilibria of unfractionated hemolysates from a normal adult (○----○) and from a heterozygote for hemoglobin Broussais (●-·-·-●), and those of hemoglobins A (○——○) and Broussais (●——●) isolated from the same hemolysate. The data were obtained at pH 7.04, 20°C. Hemoglobin concentrations were 0.1% in 0.1 M phosphate buffer. The curves were plotted according to Hill's equation.

Differential alteration of hemoglobin A and hemoglobin Broussais by the isolation procedure is not excluded; nevertheless the slight increase in oxygen affinity appears more likely to be attributable to the properties of hemoglobin Broussais itself.

Oxygen equilibrium characteristics of hemoglobin Dhofar ($\alpha_2\beta_2^{58\,Arg}$). The hemolysate from an individual heterozygous for hemoglobin Dhofar contained approximately 50% hemoglobin Dhofar. The abnormal hemolysate showed similar oxygen equilibrium characteristics to that of an unfractionated normal hemolysate (Fig. 8).

The oxygen equilibrium curves of purified hemoglobin Dhofar and purified hemoglobin A are shown in Fig. 8. P_{50} at pH 6.95, 20°C was 8.6 mm Hg for hemoglobin Dhofar and 8.5 mm Hg for hemoglobin A. The average n value in Hill's equation was 2.69±0.04 (SD, N = 5) for hemoglobin Dhofar and 2.68±0.03 (SD, N = 5) for hemoglobin A. The Bohr effect was similar in both hemoglobins (Fig. 9). The value of P_{50} for hemoglobin A in this experiment is smaller than those obtained in the other experiments. It was assumed, therefore, that the purification procedure altered slightly the normal hemoglobin A and possibly also hemoglobin Dhofar. Nevertheless, oxygen equilibrium characteristics—oxygen affinity, n values and the Bohr effects of purified hemoglobin Dhofar and hemoglobin A were indistinguishable. Unfractionated hemolysate containing approximately 50% hemoglobin Dhofar also showed oxygen equilibrium characteristics identical with unfractionated normal hemolysate. The functional properties of hemoglobin Dhofar appeared to be similar to that of hemoglobin A.

DISCUSSION

It is assumed that most amino acid residues at the external surface of the hemoglobin molecule do not influence the function of hemoglobin (7) and consequently their replacement would not affect the functional properties of the molecule. Many mutant hemoglobins with amino acid substitutions occurring at the external surface of the molecule exhibit normal oxygen equilibrium characteristics, with the exception of a few variants (27–30).

Both hemoglobins Broussais and L Ferrara have an

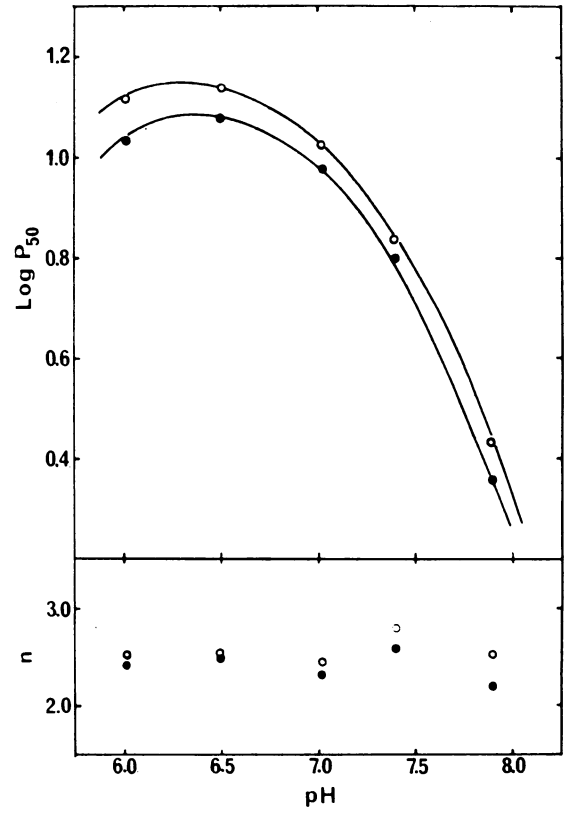

FIGURE 7 pH dependence of oxygen affinity and values of Hill constant n for hemoglobin A (○) and hemoglobin Broussais (●).

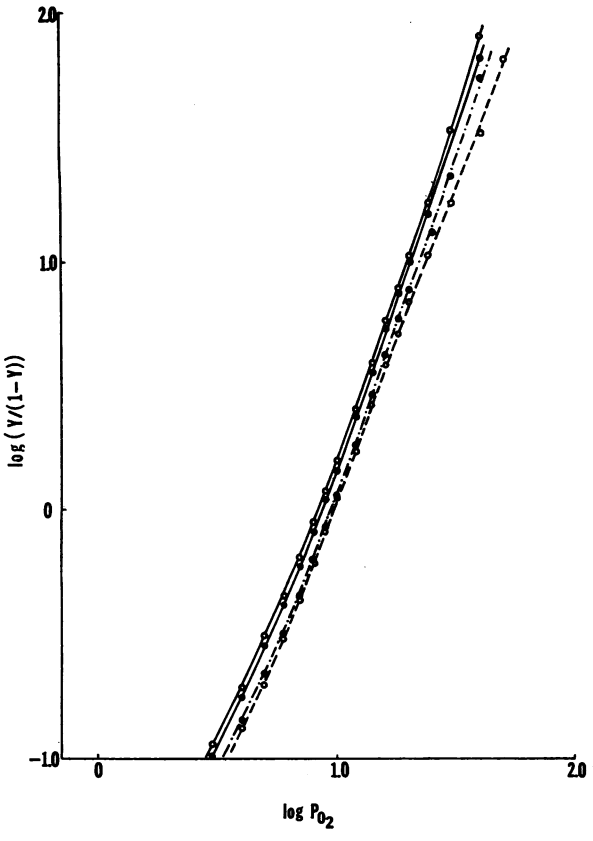

FIGURE 8 Oxygen equilibria of unfractionated hemolysates from a normal adult (○----○) and from a heterozygote for hemoglobin Dhofar (●-·-·-●), and those of hemoglobins A (○——○) and Dhofar (●——●) isolated from the same hemolysate. The data were obtained at pH 6.95, 20°C. Hemoglobin concentrations were 0.1% in 0.1 M phosphate buffer. The curves were plotted according to Hill's equation.

amino acid substitution at the external surface of the hemoglobin molecule. They showed slightly elevated oxygen affinity with normal heme-heme interaction and normal Bohr effect. X-ray crystallography analysis at 5.5 A resolution (3) suggested that in hemoglobin A the side chain of lysine at FG2(90)α may form a salt bridge with the propionic acid side chain of heme, although this hypothesis could not be confirmed by analysis at 2.8 A resolution (6). The lysine at FG2(90)α is replaced by asparagine in hemoglobin Broussais. The mechanism by which the amino acid substitution at this site in hemoglobin Broussais alters the oxygen affinity of the molecule is not clear. However, lysine at FG2(90)α is an invariant residue in all mammalian hemoglobins whose primary structures have been determined (7). This residue may have some special role on the functional properties of hemoglobin.

Nagel, Ranney, Bradley, Jacobs, and Udem (31) reported that hemoglobin L Ferrara had almost normal functional properties, and had a P_{50} value smaller than that of hemoglobin A by 21% at pH 7.4, 10°C. In the present study, values of P_{50} in hemoglobin L Ferrara were 23–31% smaller than those of hemoglobin A. This difference between hemoglobin A and hemoglobin L Ferrara is significant. We concluded, therefore, that hemoglobin L Ferrara has a higher oxygen affinity than hemoglobin A.

In hemoglobin L Ferrara the amino acid substitution involves the site of CD5(47) of the α-chain. On the CD corner of the α-chain in hemoglobin A amino acid residues at CD1(43), CD3(45) and CD4(46) have contact with heme but aspartic acid at CD5(47) is located at the external suface of the hemoglobin molecule and has no contact with heme or other subunits. The oxygen affinity of hemoglobin increases when this aspartic acid is replaced by glycine in hemoglobin L Ferrara. Nagel et al. (31) also reported that hemoglobin L Ferrara showed heat unstability at 55°C or higher temperature. In a polypeptide chain, glycine is permitted conformations which are forbidden to aspartic acid or other amino acids (32).

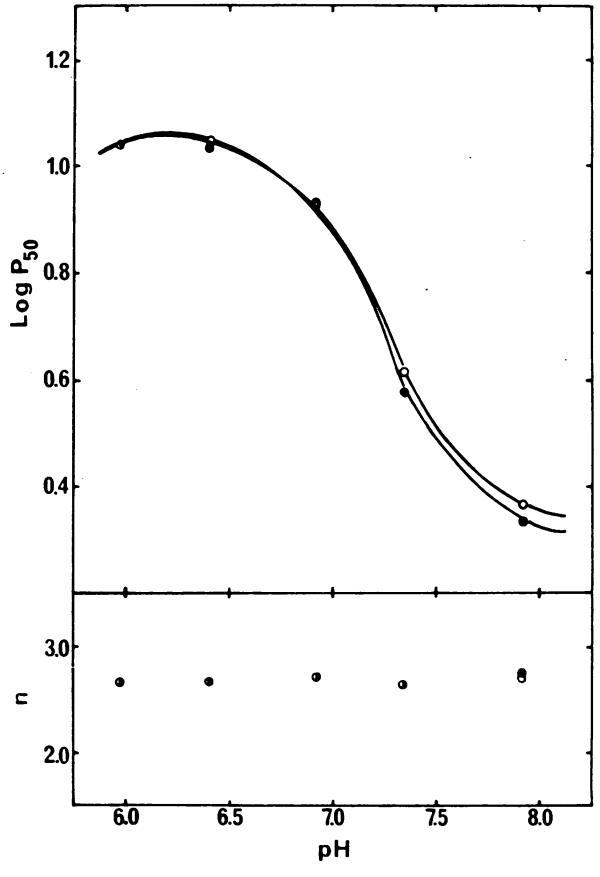

FIGURE 9 pH dependence of oxygen affinity and values of Hill constant n for hemoglobin A (○) and hemoglobin Dhofar (●).

TABLE I

Functional Properties of Abnormal Hemoglobins with Amino Acid Substitution at α_1-β_2-Contact

Designation	Amino acid substitution	Oxygen affinity	Heme-heme interaction	Bohr effect	Reference
J Cape town	FG4(92)α Arg → Gln	High	Diminished	Normal	38
Chesapeake	FG4(92)α Arg → Leu	High	Diminished	Normal	36
G Georgia	G2(95)α Pro → Leu	High	Diminished	Reduced	40
Rampa	G2(95)α Pro → Ser	High	Diminished	Reduced	40
Yakima	G1(99)β Asp → His	High	Diminished	Normal	39
Kempsey	Gl(99)β Asp → Asn	High	Diminished	Normal	37
Kansas	G4(102)β Asn → Thr	Low	Diminished	Normal	41
Hirose	C3(37)β Try → Ser	High	Diminished	Reduced	This paper

In hemoglobin L Ferrara, therefore, the conformation of the CD corner of the α-chain may be different from that of hemoglobin A, resulting in a change of heme function and unstability of structure.

It is of interest that in hemoglobin Hasharon the replacement of aspartic acid at CD5(47)α by histidine makes the structure of the mutant hemoglobin molecule unstable (33, 34). The present data in regards to the high oxygen affinity of hemoglobin L Ferrara and structural unstability of hemoglobins L Ferrara and Hasharon indicate that aspartic acid at CD5(47)α in hemoglobin A plays an important role in maintenance of normal structure and function of the hemoglobin molecule, although the atomic model of this position does not indicate this.

In hemoglobin Dhofar proline at E2(58)β is replaced by arginine. Proline residues play an important role in the conformation of the helical region of the molecule (2, 32). In hemoglobin Dhofar the amino acid substitution occurs at the helix E which participates in the formation of the heme pocket and which contains the distal histidine. It could be expected, therefore, that substantial functional changes would be induced by the substitution of arginine for proline. Contrary to our expectation, hemoglobin Dhofar does not differ significantly from hemoglobin A in oxygen affinity, heme-heme interaction, or the Bohr effect. During oxygenation of hemoglobin, the distance between the porphyrin and helix E is widened to make room for the oxygen molecule (35). It can be argued that since the E2(58)β position is near the corner of helix E at the outer surface of the molecule and arginine can be thus permitted to extend its long side chain externally without disturbing neighboring residues or subunits, the amino acid substitution does not effect the movement of the helix E and, thus, the functional properties of the molecule remain unaltered.

Hemoglobin Hirose involves an amino acid substitution at the α_1-β_2-contact: tryptophan at C3(37)β is replaced by serine. Tryptophan at C3(37)β in hemoglobin A has contacts with five amino acid residues of the α-chain including FG4(92)αArg, FG5(93)αVal, G1(94)αAsp, G2(95)αPro, and HC2(140)αTyr (6). These contacts comprise 28 atoms which are more than one-third of the atoms making up the α_1-β_2-contact (6), and conceivably such contacts can not be adequately maintained in hemoglobin Hirose.

During transition from oxy-form to deoxy-form of the hemoglobin molecule, the relative displacement of the β-chain to the α-chain is greater at the α_1-β_2-contact than at the α_1-β_1-contact. The relative displacement of atoms at the former contact can be as much as 5.7 A (4, 6). Oxygen equilibrium characteristics of seven abnormal hemoglobins, in which amino acid substitutions occur at the α_1-β_2-contact, have been reported (36-41). Six of them have high oxygen affinity and one has low oxygen affinity. All of them show reduced heme-heme interaction, and two of them show reduced Bohr effect (Table I). Hemoglobin Hirose also showed a reduced Bohr effect in addition to high oxygen affinity and diminished heme-heme interaction.

In hemoglobin Hirose, breakdown of contact between tryptophan at C3(37)β and tyrosine at HC2(140)α seems chiefly to account for the functional disturbance.

The importance of the α_1-β_2-contact in the transmission of heme-heme interaction has been pointed out by several investigators (6, 7). Briehl and Hobbs (42) suggested on the basis of ultraviolet spectrum studies of hemoglobin that tryptophan at C3(37)β played an important role on interchain interaction. Perutz (35) also showed that contact between tryptophan at C3(37)β and tyrosine at HC2(140)α took part in conformational change of β-chain from oxy-form to deoxy-form. The breakdown of this contact may disturb the conformational change of the β-chain during the oxygenation-deoxygenation reaction, resulting in decreased heme-heme interaction and high oxygen affinity.

The C-terminal histidine of the β-chain contributes to 50% of the Bohr effect (43) and α-amino groups of the α-chain contributes to another 25% of the Bohr effect (44). The sites or amino acid residues

which may contribute to the remaining one-quarter of the Bohr effect have still not been identified precisely. Tryptophan at C3(37)β has no direct contact with those residues responsible for the Bohr effect. Since tyrosine at HC2(140)α contributes to the liberation of Bohr proton bound to valine at NA1(1)α (35), breakdown of contact between tryptophan at C3(37)β and tyrosine at HC2(140)α may influence the release of Bohr proton by changes in quarternary conformation in the transition from deoxy-form to oxy-form. This can not explain all of the decreased Bohr effect in hemoglobin Hirose. Hemoglobin Hirose moves more slowly than hemoglobin A on starch gel electrophoresis at pH 8.6, although tryptophan and serine are both neutral amino acids. It is likely that the amino acid replacement in hemoglobin Hirose affects not only the α_1-β_2-contact but also the steric conformation of the molecule.

It is the general impression that high oxygen affinity of structural variants other than unstable hemoglobins usually leads to erythrocytosis (36–40) and low oxygen affinity results in reduced hemoglobin concentration in the peripheral blood (15, 29). In spite of the obvious abnormality in oxygen equilibrium characteristics, no specific hematological and clinical findings due to the abnormal hemoglobins were observed in the cases heterozygous for hemoglobin L Ferrara or hemoglobin Hirose.

In hemoglobin L Ferrara and hemoglobin Broussais, the change in oxygen affinity is slight and the proportion of the abnormal hemoglobins in hemolysates is quite small. Unfractionated hemolysates from these individuals have, therefore, oxygen equilibrium characteristics similar to that of unfractionated normal hemolysates. The carriers of these abnormal hemoglobins do not show clinical or hematological manifestations resulting from the abnormal hemoglobins.

Hemoglobin Hirose is very aberrant in all its oxygen equilibrium characteristics, despite the fact that no abnormal hematologic findings were demonstrable. The oxygen equilibrium curve of an unfractionated hemolysate, containing approximately 40% hemoglobin Hirose, was biphasic showing a very high oxygen affinity at low oxygen tension, whereas the upper part of the oxygen equilibrium curve resembled that of hemoglobin A in shape and position.

In the absence of an interaction between hemoglobin A and a high oxygen affinity mutant, the oxygen equilibrium curve of the hemolysate containing these two components should reflect the presence of the abnormal hemoglobin at low oxygen tension and hemoglobin A at high oxygen tension. If the two hemoglobins are not independent in reacting with oxygen, the entire oxygenation curve should be shifted to the left of hemoglobin A (45). Hemoglobin Hirose corresponds to the former case (Fig. 3), and under physiological conditions, that is, above 75% oxygen saturation of blood (46), hemoglobin A chiefly contribute to exchange of oxygen. Because the carriers of hemoglobin Hirose show no hematological abnormality, there must exist some way of compensating for the disturbed heme function other than increase in erythropoiesis.

As seen in the present cases, structural and functional aberrations resulting from molecular alteration do not always seem to correspond with the quality and the quantity of clinical manifestations. The following factors are all involved with the manifestation of clinical symptoms resulting from disturbed heme function: (*a*) magnitude of change in oxygen affinity of hemoglobin, (*b*) proportion of abnormal hemoglobin in the hemolysate, (*c*) interaction between normal and abnormal hemoglobins in the heterozygous state during interaction with oxygen, and (*d*) reactivity of abnormal hemoglobin with organic phosphates prescent in erythrocytes, such as 2,3-diphosphoglycerate (DPG). The latter factor has been shown to affect the oxygen affinity of hemoglobin (47, 48). Hemoglobin A has higher oxygen affinity than hemoglobin F, but in the presence of DPG, the comparative oxygen affinities are reversed (49). If an amino acid substitution alters the affinity of hemoglobin for DPG, then the hemoglobin will behave in a different way "in vivo" as compared with "in vitro" where DPG or other organic phosphates are not present. The effect of DPG or other organic phosphates on function of hemoglobin Hirose has not yet been clarified. Further study is necessary to interpret the reasons for the normal hematological findings in the presence of the disturbed heme function in individuals heterozygous for hemoglobin Hirose.

ACKNOWLEDGMENTS

The author wishes to express his thanks to Professor T. Yanase, Dr. M. Hanada, and Dr. Y. Ohta of Kyushu University for their continuous support and advice; to Dr. K. Imai of Osaka University for arrangement of the instrument assembly for oxygen equilibrium studies; to Dr. H. B. Hamilton of Atomic Bomb Casualty Commission in Hiroshima for his valuable suggestions; and to Dr. H. Cleve and Dr. S. D. Litwin of Cornell University for their careful reading of this manuscript. The author is indebted to Dr. T. Kumamoto of Moji Hospital of Japanese National Railway Corporation, Dr. T. Fujimura, Dr. K. Kawasaki, and Dr. K. Yamaoka of Kyushu University for help in obtaining the blood samples.

REFERENCES

1. Pauling, L., H. A. Itano, S. J. Singer, and I. C. Wells. 1949. Sickle cell anemia, a molecular disease. *Science (Wash. D. C.)*. **110**: 543.
2. Perutz, M. F. 1965. Structure and function of hemoglobin. I. A tentative atomic model of horse oxyhemoglobin. *J. Mol. Biol.* **13**: 646.

3. Perutz, M. F., J. C. Kendrew, and H. C. Watson. 1965. Structure and function of hemoglobin. II. Some relations between polypeptide chain configuration and amino acid sequence. *J. Mol. Biol.* **13**: 669.
4. Muirhead, H., J. M. Cox, L. Mazzarella, and M. F. Perutz. 1967. Structure and function of hemoglobin. III. A three-dimensional Fourier synthesis of human deoxyhemoglobin at 5.5 Å resolution. *J. Mol. Biol.* **28**: 117.
5. Bolton, W., J. M. Cox, and M. F. Perutz. 1968. Structure and function of hemoglobin. IV. A three-dimensional Fourier synthesis of horse deoxyhemoglobin at 5.5 Å resolution. *J. Mol. Biol.* **33**: 283.
6. Perutz, M. F., H. Muirhead, J. M. Cox, and L. C. G. Goaman. 1968. Three-dimensional Fourier synthesis of horse oxyhemoglobin at 2.8 Å resolution: the atomic model. *Nature (Lond.)*. **219**: 131.
7. Perutz, M. F., and H. Lehmann. 1968. Molecular pathology of human hemoglobin. *Nature (Lond.)*. **219**: 902.
8. Hanada, M., M. Seita, I. Ohya, and K. Yamaoka. 1963. Studies on abnormal hemoglobins. (II) The structural abnormalities of hemoglobin Shimonoseki, hemoglobin Kokura and hemoglobin Umi. *Proc. Symp. Chem. Physiol. Pathol.* **3**: 136.
9. Hanada, M., Y. Ohta, T. Imamura, T. Fujimura, K. Kawasaki, K. Yamaoka, and M. Seita. 1964. Studies on abnormal hemoglobins in western Japan. *Jap. J. Hum. Genet.* **9**: 253.
10. Imamura, T. 1966. Hemoglobin Kagoshima: an example of hemoglobin Norfolk in a Japanese family. *Am. J. Hum. Genet.* **18**: 584.
11. Ohta, Y., T. Imamura, T. Fujimura, K. Kawasaki, K. Yamaoka, and M. Hanada. 1967. A chemical abnormality in hemoglobin Miyada from Japanese individuals. *Jap. J. Hum. Genet.* **12**: 127.
12. Kawasaki, K., Y. Ohta, T. Imamura, and M. Hanada. 1967. Characterization of hemoglobin Yukuhashi. *Jap. J. Clin. Hematol.* **8**: 175.
13. Fujimura, T., Y. Ohta, T. Imamura, K. Kawasaki, K. Yamaoka, and M. Hanada. 1967. Characterization of hemoglobin Tagawa-I. *Acta Haematol. Jap.* **30**: 639.
14. Yamaoka, K. 1971. Hemoglobin Hirose $\alpha_2\beta_2$37(C3) tryptophan yielding serine. *Blood J. Hematol.* **38**: 730.
15. Imamura, T., S. Fujita, Y. Ohta, M. Hanada, and T. Yanase. 1969. Hemoglobin Yoshizuka (G10(108)β asparagine $\rightarrow$ aspartic acid): a new variant with a reduced oxygen affinity from a Japanese family. *J. Clin. Invest.* **48**: 2341.
16. Lehmann, H., and R. W. Carrell. 1969. Variations in the structure of human hemoglobin. With particular reference to the unstable hemoglobins. *Br. Med. Bull.* **25**: 14.
17. de Traverse, P. M., H. Lehmann, M. L. Coquelet, D. Beale, and W. A. Isaacs. 1966. Etude d'une hemoglobine Jα non encore decrite, dans une familie francaise. *C. R. Soc. Biol.* **160**: 2270.
18. Marengo-Rowe, A. J., P. A. Lorkin, E. Gallo, and H. Lehmann. 1968. Hemoglobin Dhofar—a new variant from Southern Arabia. *Biochim. Biophys. Acta.* **168**: 58.
19. Drabkin, D. L. 1946. Spectrophotometric studies. XIV. The crystallographic and optical properties of the hemoglobin of man in comparison with those of other species. *J. Biol. Chem.* **164**: 703.
20. Kunkel, H. G., and G. Wallenius. 1955. New hemoglobin in normal adult blood. *Science (Wash. D. C.)*. **122**: 288.
21. Huisman, T. H. J., and A. M. Dozy. 1965. Studies on the heterogeneity of hemoglobin. IX. The use of tris(hydroxymethyl) aminomethane-HCl buffers in the anion-exchange chromatography of hemoglobins. *J. Chromatogr.* **19**: 160.
22. Paul, K. G., H. Theorell, and A. Akeson. 1953. The molar light absorption of pyridine ferroprotoporphyrin (pyridine hemochromogen). *Acta Chem. Scand.* **7**: 1284.
23. Imai, K., H. Morimoto, M. Kotani, H. Watari, W. Hirata, and M. Kuroda. 1970. Studies on the function of abnormal hemoglobins I. An improved method for automatic measurement of the oxygen equilibrium curve of hemoglobin. *Biochim. Biophys. Acta.* **200**: 189.
24. Benesch, R., G. Macduff, and R. E. Benesch. 1965. Determination of oxygen equilibria with a versatile new tonometer. *Anal. Biochem.* **11**: 81.
25. Bohr, C., K. Hasselbal, and A. Krogh. 1904. Ueber einen in biologischer Beziehung wichtigen Einfluss, den die Kohlen-saurespannung des Blutes auf dessen Sauerstoffbindung uebt. *Skand. Arch. Physiol.* **16**: 402.
26. Hill, A. V. 1910. The possible effects of the aggregation of the molecules of hemoglobin on its dissociation curves. *J. Physiol. (Lond.)*. **40**: P4.
27. Bookchin, R. M., R. L. Nagel, and H. M. Ranney. 1967. Structure and properties of hemoglobin C Harlem, a human hemoglobin variant with amino acid substitutions in 2 residues of the β-polypeptide chain. *J. Biol. Chem.* **242**: 248.
28. Huisman, T. H. J., J. Still, and C. M. Nechtman. 1963. The oxygen equilibria of some slow-moving human hemoglobin types. *Biochim. Biophys. Acta.* **74**: 69.
29. Stamatoyannopoulos, G., J. T. Parer, and C. A. Finch. 1969. Physiologic implications of a hemoglobin with decreased oxygen affinity (hemoglobin Seattle). *N. Engl. J. Med.* **281**: 915.
30. Imai, K., H. Morimoto, M. Kotani, S. Shibata, T. Miyaji, and K. Matsutomo. 1970. Studies on the function of abnormal hemoglobins. II. Oxygen equilibrium of abnormal hemoglobins: Shimonoseki, Ube II, Hikari, Gifu and Agenogi. *Biochim. Biophys. Acta.* **200**: 197.
31. Nagel, R. L., H. M. Ranney, T. B. Bradley, A. Jacobs, and L. Udem. 1969. Hemoglobin L Ferrara in a Jewish family associated with a hemolytic state in the propositus. *Blood J. Hematol.* **34**: 157.
32. Ramakrishnan, C., and G. N. Ramachandran. 1965. Stereochemical criteria for polypeptide and protein chain conformations. II. Allowed conformations for a pair of peptide units. *Biophys. J.* **5**: 909.
33. Halbrecht, I., W. A. Isaacs, H. Lehmann, and F. Ben-Porat. 1967. Hemoglobin Hasharon(α47 Aspartic acid $\rightarrow$ Histidine). *Isr. J. Med. Sci.* **3**: 827.
34. Charache, S., A. M. Mondzac, U. Gessner, and E. E. Gayle. 1969. Hemoglobin Hasharon(α_2^{47His}(CD5)β_2): a hemoglobin found in low concentration. *J. Clin. Invest.* **48**: 834.
35. Perutz, M. F. 1970. Stereochemistry of cooperative effects in hemoglobin. *Nature (Lond.)*. **228**: 726.
36. Charache, S., D. J. Weatherall, and J. B. Clegg. 1966. Polycythemia associated with a hemoglobinopathy. *J. Clin. Invest.* **45**: 813.
37. Reed, C. S., R. Hampson, S. Gordon, R. T. Jones, M. J. Novy, B. Brimhall, M. J. Edwards, and R. D. Koler. 1968. Erythrocytosis secondary to increased oxygen affinity of a mutant hemoglobin, Hemoglobin Kempsey. *Blood J. Hematol.* **31**: 623.

38. Lines, J. G., and R. McIntosh. 1967. Oxygen binding by hemoglobin J-Cape town($\alpha_2$92 Arg $\rightarrow$ Gln). *Nature* (*Lond.*). **215**: 297.
39. Novy, M. J., M. J. Edwards, and J. Metcalfe. 1967. Hemoglobin Yakima. II. High blood oxygen affinity associated with compensatory erythrocytosis and normal hemodynamics. *J. Clin. Invest.* **46**: 1848.
40. Smith, L. L., C. F. Plese, B. P. Barton, S. Charache, J. B. Wilson, and T. H. J. Huisman. 1972. Subunit dissociation of the abnormal hemoglobins G Georgia (α_2^{95Leu}(G2)β_2) and Rampa(α_2^{95Ser}(G2)β_2). *J. Biol. Chem.* **247**: 1433.
41. Bonaventura, J., and A. Riggs. 1968. Hemoglobin Kansas, a human hemoglobin with a neutral amino acid substitution and an abnormal oxygen equilibrium. *J. Biol. Chem.* **243**: 980.
42. Briehl, R. W., and J. F. Hobbs. 1970. Ultraviolet difference spectra in human hemoglobin. I. Difference spectra in hemoglobin A and their relation to the function of hemoglobin. *J. Biol. Chem.* **245**: 544.
43. Perutz, M. F., H. Muirhead, L. Mazzarella, R. A. Crowther, J. Greer, and J. V. Kilmartin. 1969. Identification of residues responsible for the alkaline Bohr effect in hemoglobin. *Nature* (*Lond.*). **222**: 1240.
44. Kilmartin, J. V., and L. Rossi-Bernardi. 1969. Inhibition of CO_2 combination and reduction of the Bohr effect in hemoglobin chemically modified at its α-amino groups. *Nature* (*Lond.*). **222**: 1243.
45. Bellingham, A. J., and E. R. Huehns. 1968. Compensation in hemolytic anemias caused by abnormal hemoglobins. *Nature* (*Lond.*). **218**: 924.
46. Bartels, H., R. Beer, E. Fleischer, H. J. Hoffheinz, J. Krall, G. Rodewald, J. Wenner, and I. Witt. 1955. Bestimmung von Kurzschlussdurchblutung und Diffusionskapazitaet der Lunge bei Gesunden und Lungen Kranken. *Pfluegers Arch. Gesamte Physiol. Menschen Tiere.* **261**: 99.
47. Benesch, R., and R. E. Benesch. 1967. The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin. *Biochem. Biophys. Res. Commun.* **26**: 162.
48. Chanutin, A., and R. R. Curnish. 1967. Effect of organic and inorganic phosphates on the oxygen equilibrium of human erythrocytes. *Arch. Biochem. Biophys.* **121**: 96.
49. Tyuma, I., and K. Shimizu. 1970. Effect of organic phosphates on the difference in oxygen affinity between fetal and adult human hemoglobin. *Fed. Proc.* **29**: 1112.