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A Sensitive Method for Detecting Subunit Dissociation of Abnormal Hemoglobin

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Abstract Dissociation of abnormal hemoglobin is compared with normal hemoglobin (Hb A) by gel permeation employing Sephadex G-75 column. Equimolar solutions (5 μM) of Hb A and an abnormal hemoglobin in 0.01 M phosphate buffer, pH 7.00 containing 0.09 M NaCl and a trace amount of KCN, are introduced into the column (1.0×60 cm, upward flow) by a peristaltic pump (flow rate 0.25 ml/min) one after another. The absorbance at 280 nm or Soret band of the effluent is continuously monitored. The difference in the degree of dissociation is detected by the change in the absorbance at the interface where the two hemoglobins meet. The method developed here has been applied to fifteen different abnormal hemoglobins; twelve of them (80%) have shown either accelerated or reduced dissociation. The method is highly sensitive and specific for the detection of abnormal hemoglobins.

Key Werms: Abnormal hemoglobin, Subunit dissociation

Introduction

Hemoglobin is a tetrameric molecule consisting of two pairs of α and non-α subunits .Hemoglobin in adult blood consists mostly of Hb A ($\alpha_2\beta_2$), a few percentages of Hb A_2 ($\alpha_2\delta_2$) and a trace amount of Hb F ($\alpha_2\gamma_2$)[1]. The four subunits in a hemoglobin molecule mutually interact by noncovalent bonds, i. e. hydrogen bonds, hydrophobic forces, van der Waals potentials and salt bridges. In a Hb A molecule, a given α chain (α_1) have three interfaces with other subunits. The α_1 subunit shares extensive, tight contacts with one of the β chains (β_1) ,while it has more flexible interactions with another β chain (β_2). The contacts between like chains, i.e. $\alpha_1\alpha_2$ and $\beta_1\beta_2$, are limited and of little importance. Therefore, a hemoglobin tetramer is appropriately treated as a dimer of $\alpha_1\beta_1$ units, i.e. $(\alpha\beta)_2$[2]. In a highly diluted solution, the tetrameric molecule commences to dissociate into a pair of $\alpha\beta$ subunits[3]. The $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) contact is believed to play a major role in the dissociation, and in fact the dissociation is a well known property of the abnormal hemoglobins with amino acid substitution at the $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) interface[4]. The methods detecting the subunit dissociation are usually carried out by determination of the mean molecular weight by ultracentrifugation[5-7] or by gel

permeation chromatography[8-9]. These techniques are elaborate and annoyous as well as of low sensitivity. In 1971 Rosa et al[10] detected the increased subunit dissociation of Hb Toulouse relative to Hb A. However, their method is not established, and has been ignored. Here, we have improved their method and have applied it to a number of other abnormal hemoglobins.

Materials and Methods

Heparinized blood was obtained by venipuncture, hemolysed and stored in the CO form at 4°C until the analysis. The CO hemoglobin was converted to O_2 hemoglobin by flash photolysis before use. Gel permeation study was performed in the oxy or cyanmet hemoglobin (HiCN) form. Solutions of Hb A and abnormal hemoglobin at exactly the same concentration (about 5 μM) were prepared in 0.01 M phosphate buffer, pH 7.00 (containing 0.90 M NaCl and a trace amount of KCN). The hemoglobin solution of 5 μM is attained by adjusting the absorbance of the hemoglobin solution to 0.655 at 415 nm and 0.613 at 421 nm in oxy and HiCN forms, respectively. These values were calculated by the millimolar extinction coefficient of the oxyhemoglobin (131) and HiCN (122.5) at their Soret bands, i.e. 415 nm in oxy form and 421 nm in HiCN. The hemoglobin solutions were led to a Sephadex G-75 column (1.0×60 cm) from the bottom through a peristaltic pump (flow rate: 0.25 ml/min). The effluent from the top of the column was introduced to the flow cell photometer. The absorption at 280 nm or of the Soret band was continuously recorded (Fig. 1). The column and hemoglobin solutions in the reservoirs were kept at 8°C throughout the experiment. The column was first equilibrated with the Hb A solution, then the abnormal hemoglobin was started to flow successively to Hb A by switching the 4-way valve to the abnormal hemoglobin solution. The method is called "dissociation test" in this paper.

Principle

The absorbances of Hb A and abnormal hemoglobin are equal as long as their concentrations and derivative forms are the same. In the dissociation test described above, the absorbance of the effluent will give a flat "base line" (Fig. 2a). In a

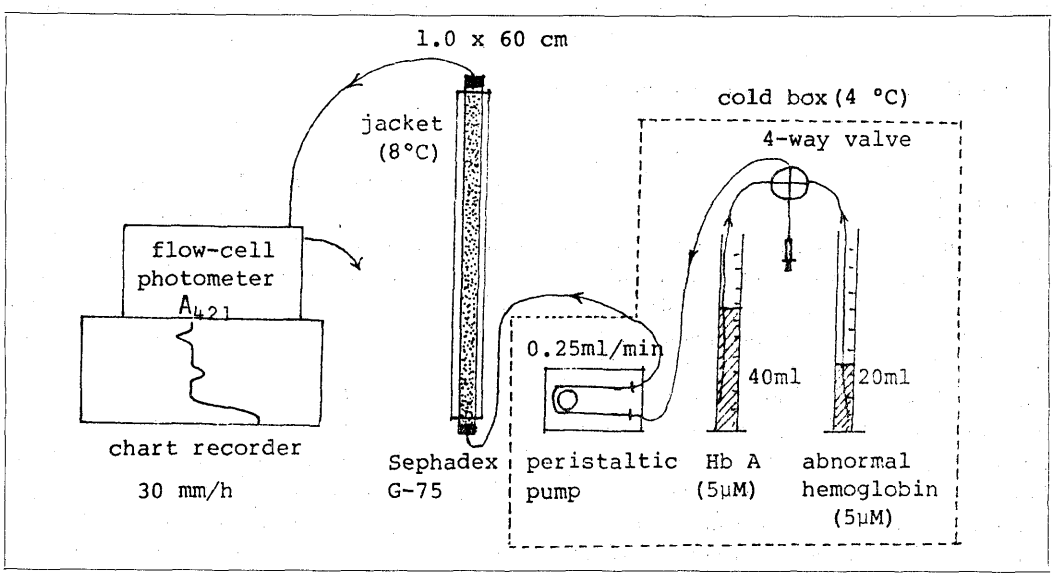

Fig. 1 Equipment and methods for subunit dissociation of hemoglobin At least 20 and 40 ml of 5 μM abnormal and a control (Hb A) hemoglobin solution, respectively, are required. Through a 4-way valve, they are successively introduced into a Sephadex G-75 column by a peristaltic pump.

highly diluted solution, e.g. 5μM, even Hb A is slightly dissociated into dimers[4]. If the abnormal hemoglobin dissociates more readily than Hb A, the abnormal hemoglobin elutes slightly slower than the normal hemoglobin (Hb A) in the gel permeation. This causes a downward deflection of the absorbance from the base line absorption at the interface between the foregoing Hb A and the following abnormal hemoglobin. Owing to equilibration between the dissociated (dimer) and undissociated (tetramer) forms, as well as diffusing effect of gel permeation, a smooth downward deflection is actually observed (Fig. 2b). Thus, deflection of the base line reflects the degree of dissociation of the abnormal hemoglobin relative to Hb A (relative dissociation). If the abnormal hemoglobin dissociates less readily than Hb A, an upward deflection will be obtained.

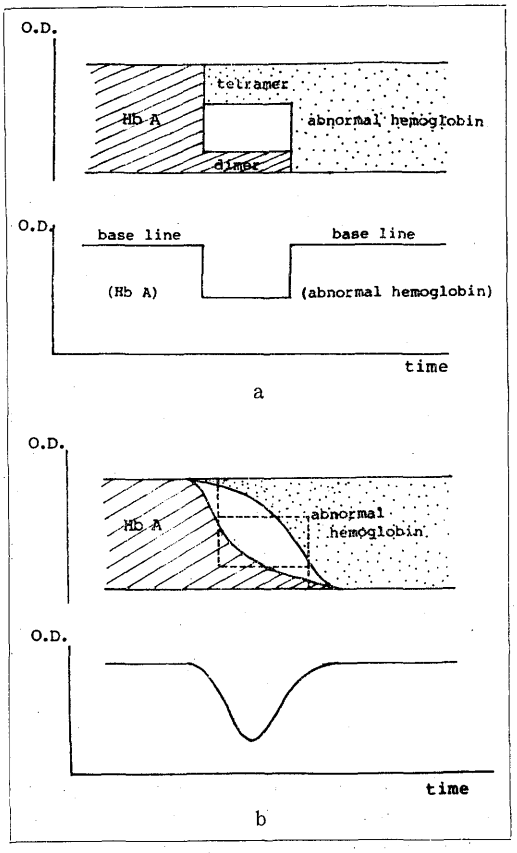

Fig. 2 Principles of the subunit dissociation test
a) Schematic representation of an idealized elution pattern at the interface. A portion of Hb A dissociates into dimer which elutes later than tetramer. Whereas, if a larger portion of the abnormal hemoglobin dissociates into dimer, the sum of the concentration of the dimeric form of Hb A and the tetrameric form of abnormal hemoglobin at their interface becomes less than the original concentration of them (base line).
b) Actual elution pattern at the interface
The tetrameric and dimeric forms exist only in a reversible equilibrium. This generates a gradual gradient of hemoglobin concentration at the interface of the two hemoglobins, which is recorded as a smooth V-shaped depression from the base line.

Results

A dissociation profile of Hb Hirose is shown in Fig. 3. Hb Hirose is famous for its complete dissociation into $\alpha\beta$ dimers. As predicted, there is a sharp downward deflection of absorbance from the base line at the interface which is formed by the tail of Hb A and the head of Hb Hirose. When the switch is further turned back to Hb A solution, an upward deflection follows. This is because the slowly eluting dimer of Hb Hirose is now overun by the undissociated normal hemoglobin. It should be noted that both the downward and upward deflections are almost the same in shape. Suppose that the column is eluted with Hb X_2 and Hb X_1 in succession in the dissociation test, the extent of dissociation of Hb X_1 relative to Hb X_2 is tentatively called "dissociation index (X_1/X_2). It is expressed as $[A-A']/A \times 100$, where A and A′ are the absorbances at the base line and at the deflection peak, respectively. A positive value reflects a downward deflection, and indicates that Hb X_1 dissociates more readily than Hb X_2. A negative value reflects an upward deflection, and indicates that Hb X_1 dissociates less readily than Hb X_2. In Fig. 3, the downward and upward deflections reflect the dissociation index (Hb Hirose/Hb A) and

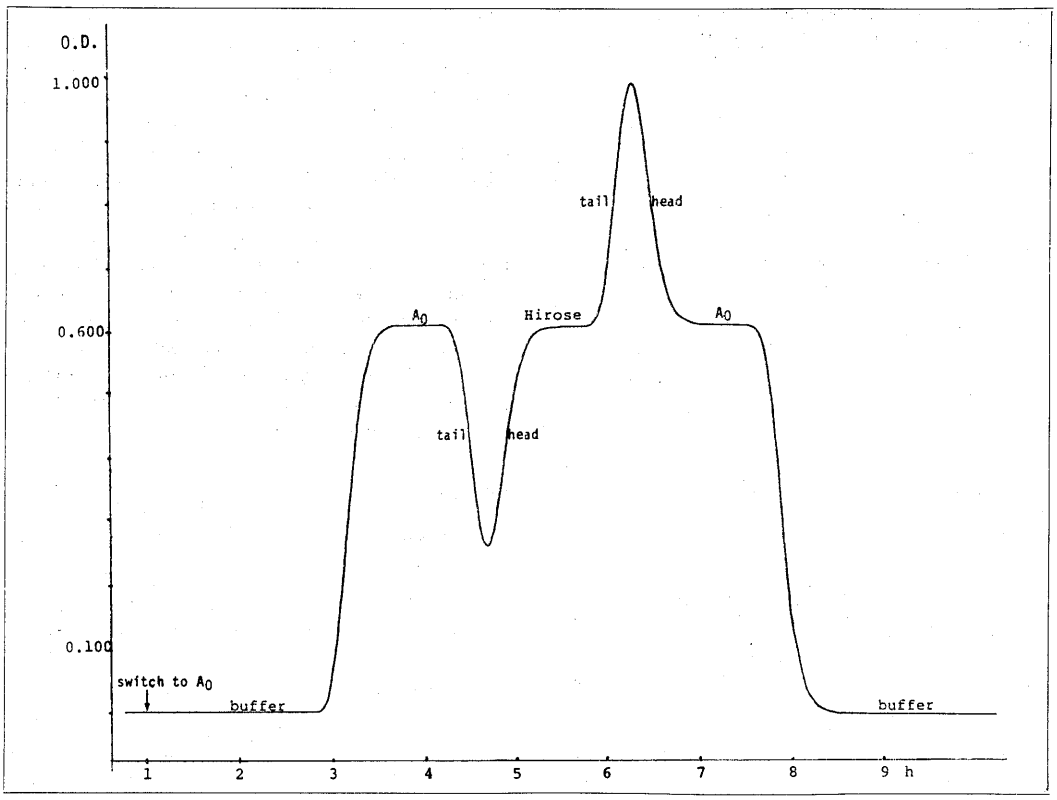

Fig. 3 Dissociation profile of Hb Hirose
A_0: Hb A, Hirose: Hb Hirose
In the downward deflection, the slowly-eluted dimer of Hb Hirose makes absorbance blank between the foregoing Hb A. In the upward deflection, the dimer now sums up to the following Hb A. The direction of the deflection depends upon the order of the hemoglobin solutions pumpted into the column.

the dissociation index (Hb A/Hb Hirose), respectively. Since the both deflections are symmetrical, the absolute values for the two dissociation indices are equal, but have opposite signs.

The dissociation index is markedly affected by flow rate. The smaller the flow rate, the greater the dissociation index (Fig. 4). For effectiveness of dissociation, the flow rate of 0.25 ml/min is selected in our experiment.

The dissociation is pH dependent (Fig. 5). At pH above 7, the higher is the pH of the elution buffer, the more effective is the dissociation. However, hemoglobin molecule (Hb A) is labile at highly alkaline pHs. Unstable hemoglobins, which are often our subjects of study, might be more labile than Hb A at alkaline pHs. Since the denaturation of the hemoglobin during the experiment must be avoided, the pH 7.0 is chosen in our dissociation test. Sephdex G-75 is more effective for the detection of subunit dissociation than Sephdex G-100 (Fig. 5). The latter has been used by Rosa in his original method[10].

Hemoglobin usually presents in oxy form. However, the formation of methemoglobin,

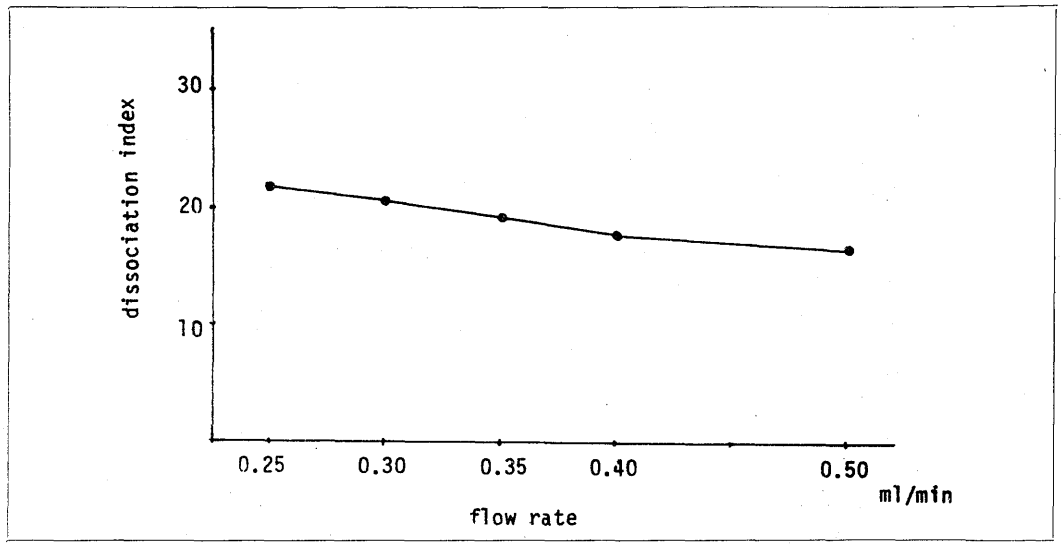

Fig. 4 Effect of flow rate on dissociation
The dissociation is most effectively observed at the smallest flow rate tested, i.e. 0.25 ml/min.

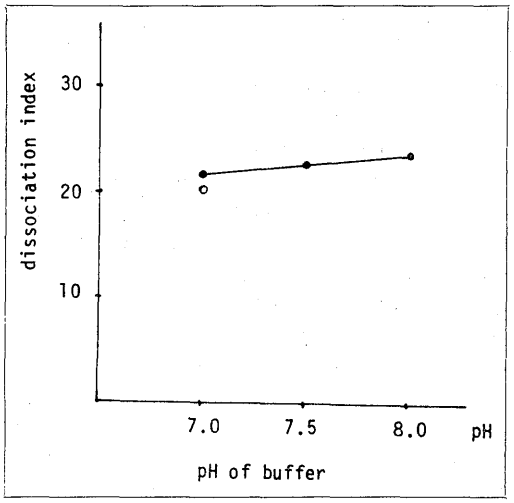

Fig. 5 pH dependency of dissociation
The dissociation index increases linearly from pH 7.0 to pH 8.0. Buffer: 0.01 M phosphate buffer, pH 7.0, 7.5 and 8.0 containing 0.09 M NaCl. flow rate : 0.25 ml/min
● : Sephadex G-75, ○ : Sephadex G-100

which is further converted to HiCN by KCN in the eluent in the dissociation test, is inevitable during storage of oxyhemoglobin or during a procedure of flash photolysis. Also, a small amount of CO hemoglobin often remains after flash photolysis. The sample (oxy form) is thus often contaminated by other derivatives. The dissociation in the three derivatives (oxy, CO and HiCN) are compared in Figure 6. The HiCN tends to dissociate more readily than the oxy or CO form. Methemoglobin formation, therefore, should be avoided as far as possible when the test is carried out in the oxy form. Such a problem does not occur, if the dissociation test is performed in the form of HiCN. HiCN is the most stable among the three derivatives of hemoglobin, and this form is especially preferred when testing unstable hemoglobins.

Hbs F and A_2 occupy less than a few percent of hemoglobin from normal adult. However, hemolysate from carriers of abnormal hemoglobin or from thalassemia traits often contain an increased amount of Hbs F

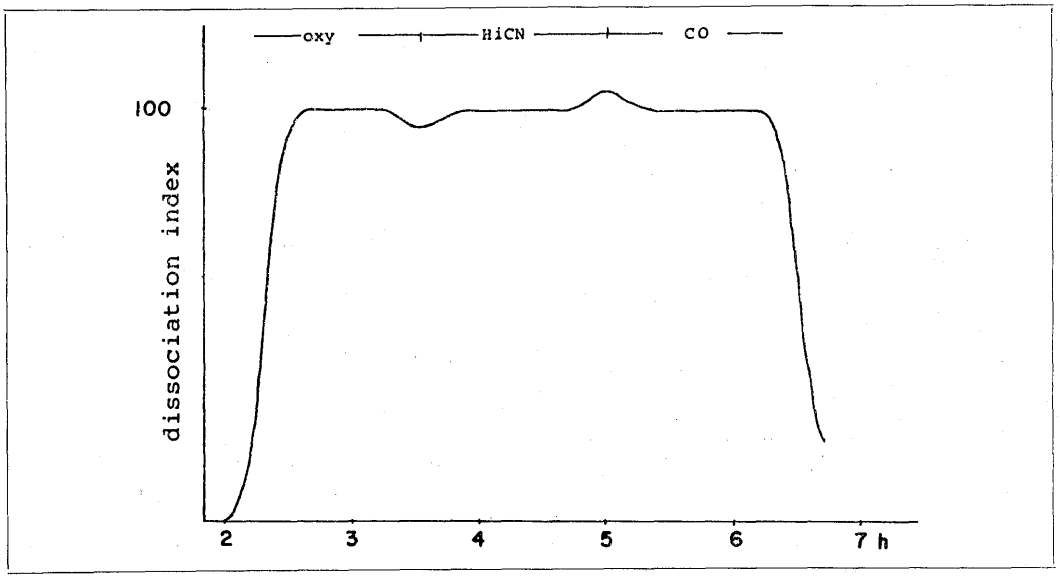

Fig. 6 Difference of dissociation between derivatives
oxy: oxyhemoglobin HiCN: cyanmethemoglobin CO: carbon monoxyhemoglobin
The profile indicates that the HiCN dissociates more readily than oxy form, and that the CO form dissociates less readily than HiCN. Since the both deflections are in the opposite direction and symmetrical, the degree of dissociation of oxy form is almost the same as CO form.

and/or A_2. Both Hbs F and A_2 dissociate slightly less than Hb A (Fig. 7). Since Hb A_2 usually comprises less than 10% of total hemoglobin in the abnormal hemolysates mentioned above, it is unlikely that Hb A_2 has a significant interference on the dissociation test of hemolysates. While, concentration of Hb F must be measured because it could be excessively increased in pathological conditions[11].

Precision of the dissociation test is given in Table 1.

The dissociation indices of abnormal hemoglobins recently analysed in our laboratory are listed in Table 2. Twelve of fifteen abnormal hemoglobins (80%) revealed an accelerated or reduced dissociation. The remaining three hemoglobins showed the same dissociation as Hb A, i.e. dissociation index of zero. All hemolysates in which an abnormal hemoglobin was not detectable by other methods also gave normal results in the dissociation test. Table 3 presents the result on four abnormal hemoglobins which were unseparable by electrophoretic method. Dissociation is increased in three, and the remaining one is detectable only by the enhanced O_2 affinity. The dissociation test is thus highly sensitive and specific for the detection of abnormal hemoglobins even if they are electrophoretically undetectable. These facts suggest that the dissociation test is useful for confirming the presence of abnormal hemoglobin in a suspected hemolysate.

Discussion

The dissociation test as described in this paper measures subunit dissociation of the sample (a purified abnormal hemoglobin or a hemolysate) relative to that of Hb A or a normal hemolysate. The purified Hb Hirose

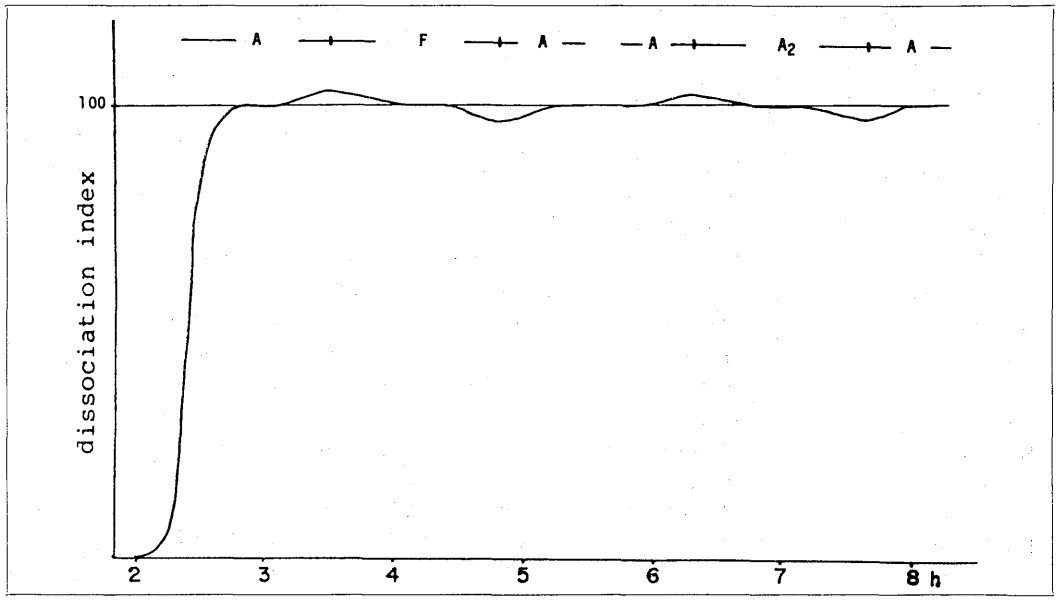

Fig. 7 Difference of dissociation between Hbs A, F and A_2

Hemolysate from a cord blood with 62.5 % Hb F (measured by alkali denaturation[12]) was used as Hb F in this test. Hbs A and A_2 are isolated from a normal hemolysate by DE-52 column chromatography[13]. The test was carried out in HiCN form and by measuring absorbance at 421 nm. The deflection at Hb A/Hb F interface would be more pronounced than that in this figure, if a pure Hb F was used. The result indicates that both Hbs F and A_2 dissociate less readily than Hb A.

Table 1 Precision of the dissociation indices

	dissociation index	
	(abn./Hb A)	(Hb A/abn.)
Hirose	64.2	
	60.0	−59.6
	57.0	−61.4
Köln (3 heme)	29.7	−29.8
	25.4	−23.2

abn.: abnormal hemoglobin,
Hb Hirose was tested in oxy form. Hb Köln was isolated in 3 heme form, and subsequently tested in HiCN form because of its instability. Dissociation indices are essentilally the same, whether the abnormal sample is introduced to the column after (abn./Hb A) or before Hb A (Hb A/abn.).

completely dissociates into dimers and stays as such, revealing a maximum deflection under the given test conditions. Most other abnormal hemoglobins are only partially dissociated, however, giving rise a deflection proportional to the extent of relative dissociation and to the purity of the paticular hemoglobin in the sample solution. Our results on various abnormal hemoglobins have proved the dissociation test highly sensitive; this test detects a very slightly increased tendency of subunit dissociation which would be missed by other methods such as ultracentrifugation and conventional gel filtration. Since both the abnormal and normal hemoglobins are in a tetramer/dimer equilibrium, a hybrid tetramer between the two hemoglobins may occur at the interface of the two hemoglobin solutions. The elution

Table 2 Dissociation indices of abnormal hemoglobins

name	dissociation index (abn/control)
Hb Iwata	6.5
Hb M Iwate	−11.7
Hb Kokura	0
Hb Utsunomiya	4.9(whole)
Hb Zürich	13.1
Hb M Saskatoon	6.2
Hb Yokohama	3.0(whole)
Hb Burke	− 7.4
Hb Hirose	55.4
Hb Köln	15.6
Hb San Diego	2.9(whole)
Hb Miyashiro	0 (whole)
Hb Saitama	4.6(whole)
Hb Toyoake	17.5
Hb Hikari	0

The signs + and − indicate the increased and decreased dissociations, respectively. The word "whole" in parenthesis denotes the sample being hemolysate, i.e. a mixture with normal hemoglobins.

Table 3 Electrophoretically silent abnormal hemoglobins and their dissociation indices (abn/control)

	dissociation index	isopropanol	O_2 affinity
Hb Yokohama	3.0(whole)	+	?
Hb Saitama	4.6(whole)	+	?
Hb Miyashiro	0 (whole)	weak	↑
Hb San Diego	2.9(whole)	−	↑

The results of isopropanol test and oxygen affinity are also presented.

pattern itself tells nothing about such a phenomenon. It is simply a reflection of the difference of the average molecular size of the hemoglobins between the two solutions.

Meticulous care must be taken for the maximum efficiency of the test. The absorbances of both the sample and control must be exactly adjusted equal to avoid misleading distortion of the base line at the interface. It is desirable to remove any dust and hemoglobin precipitate by filtering or centrifuging both the buffer and the sample prior to making final dilution of hemoglobin solution tested. Unstable hemoglobins should be examined in cyanmet form because denaturation must be avoided during experiment. Hemolysate containing a significant amount of Hb F is inappropriate as a sample, since Hb F dissociates less readily than Hb A. No air bubbles must be introduced into the colunm because the column is of upward flow.

It is suprizing that the dissociation is altered in as much as 80% of abnormal hemoglobins recently analysed in our laboratory. Moreover, the alteration is detected in abnormal hemoglobins not separable by electrophoresis which is a widely used method for screening of abnormal hemoglobin. It is estimated that more than 50% of abnormal hemoglobins are overlooked by the electrophoretical method and isopropanol test which contributes to the discovery of unstable hemoglobins by checking the instability of hemoglobin molecule[14]. Thus, majority of stable and electrophoretically silent abnormal hemoglobins remain undetected. The dissociation test is a unique method probably based upon steric distortion of $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) subunit contact induced by aminoacid substitution at various positions in the polypeptide chain. The dissociation test might become one of useful tools for survey of abnormal hemoglobins, if its time-consuming nature is improved.

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Reference

1) Wintrobe, M.M.: *Clinical Hematology*, Lea & Febiger, Philadelphia, 1981, p. 88–90.
2) Fermi, G. and Perutz, M.F.: Hemoglobin &

Myoglobin. In D.C. Phillips and F.M. Richards (eds.), *Atlas of Molecular Structures in Biology*, Clarendon Press, Oxford, 1981, p. 20-21.

3) Rosemeyer, M.A. and Huehns, E.R.: On the mechanism of the dissociation of Haemoglobin. *J. Mol. Biol.*, 25 : 253-273, 1967.

4) Sasaki, J., Imamura, T., Yanase, T., Atha, D. H., Riggs, A., Bonaventura, J. and Bonaventura, C.: Hemoglobin Hirose, A Human Hemoglobin Variant with a Substitution at the $\alpha_1\beta_2$ Interface. Subunit Dissociation and the Equilibria and Kinetics of Ligand Binding. *J. Biol. Chem.*, 253 : 87-94, 1978.

5) Hasserodt, U. and Vinograd, J.: Dissociation of Human Carbonmonoxyhemoglobin at high pH. *Proc. Nat. Acad. Sci.*, 45 : 12-15, 1959.

6) Kirshner, A. and Tanford, C.: The Dissociation of Hemoglobin by Inorganic Salts. *Biochemistry*, 3 : 291-296, 1964.

7) Kawahara, K., Kirshner, A.G. and Tanford, C.: Dissociation of human CO-Hemoglobin by urea, Guanidine Hydrochloride and other reagents. *Biochemistry*, 4 : 1203-1213, 1965.

8) Ackers, G.K. and Thompson, T.E.: Determination of Stoichiometry and Equilibrium Constants for Reversibly Associating Systems by Molecular sieve Chromatography. *Proc. Natl. Acad. Sci.*, 53 : 342-349, 1965.

9) Huehns, E.R. and Shooter, E.M.: The Properties and Reactions of Haemoglobin F and their bearing on the Dissociation Equilibrium of Haemoglobin. *Biochem. J.*, 101 : 852-860, 1966.

10) Rosa, D.C. J., Belkhodja, O. and Bierme, R.: Hemoglobin Toulouse $\alpha_2\beta_2^{66}$(E10) Lys→Glu, Structure and Consequences in Molecular Pathology. *Biochim. Biophys. Acta*, 236 : 201-207, 1971.

11) Huisman, H.J. and Jonxis, J.H.P.: *The Hemoglobinopathies*, Marcel Dekker Inc, New York and Basel, 1977, p. 28-39.

12) Betke, K., Marti, H.R. and Schlicht, I.: Estimation of Small Percentage of Foetal Haemoglobin. *Nature*, 184 : 1877-1878, 1959.

13) Abraham, E.C., Reese, A., Stallings, M. and Huisman, T.H.J.: Separation of human hemoglobins by DEAE-cellulose chromatography using glycine-KCN-NaCl developers. *Hemoglobin*, 1 : 27-44, 1976-77.

14) Ohba, Y. and Miyaji, T.: Abnormal Hemoglobins and Thalassemia Syndrome in Japan (in Japanese). *Medicina Philosophica*, 1 : 382-388, 1982.