Heat Denaturation Test for Unstable Hemoglobin, with a Note on Its Application to Hemoglobin Survey in Japan

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Shibata and his associates[1] are credited for the description of the first instance of the unstable hemoglobin hemoglobinopathy in Japan. It was in 1963 that they reported an 11 year old girl with Heinz body anemia associated with production of electrophoretically slow-moving hemoglobin. The patient had been splenectomized for the so called "hypersplenism" four years previously. The case resembled the Heinz body anemia which was seen by Scott and others[2] in Salt Lake City in 1960. However, the abnormal hemoglobin (Hb Ube-1) was unusually unstable, forming precipitation even in the process of its purification for chemical study, on account of the blockage of the active cysteine residue (β 93) on its β subunits[1].

Shortly before Shibata's communication Frick and his colleagues[3] (1962) found a Swiss family of peculiar hemoglobinopathy (Hb Zürich disease) in which Heinz body anemia is induced by medication of sulfonamide drugs. They noticed that the hemolysate prepared from the blood of their patient yielded precipitate when it was warmed at 65°C. Such a phenomenon was not seen with the hemolysates of normal subjects. This was therefore thought to be an evidence for the melecular instability of the abnormal hemoglobin contained in the patient's hemolysate. Since then a considerable number of unstable hemoglobins have been demonstrated in congenital Heinz body anemias. Hb Köln[4], Hb Genova[5], Hb Seattle[6], Hb Sidney[7], Hb Bibba[8], Hb Torino[9], Hb Hammersmith[10], Hb Sabine[11] and Hb Gun Hill[12] are such examples.

In 1964 Dacie and his associates[13] evaluated for the first time the heat lability of the hemolysate of Frick[3] as a significant clinical sign of Heinz body anemias caused by unstable hemoglobins, and published their procedure for heat denaturation test, in which hemolysate was warmed at 50°C for 1 to 3 hours to observe hemoglobin precipitate. Employing their method they could successfully detect

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the presence of unstable hemoglobins in the hemolysate which appeared to be normal by electrophoresis.

The essential character of abnormal hemoglobins consists in the amino acid substitution in the α or the non-α subunits composing their globin molecule. The substitution may be of acid or basic amino acid for neutral amino acid (or vice versa) that results in change in the negative electric charge of hemoglobin molecule, thus enabling us their electrophoretical demonstration. With few exceptions all the abnormal hemoglobins so far discovered belong to this category. However, it is expected that the substitution may be of neutral amino acid for other neutral amino acid, which does not cause appreciable change in electric charge. Electrophoresis is not helpful for the demonstration of this kind of abnormal hemoglobins. Theoretically 2151 variants are conceivable from the possible amino acid substitution on the α and the non-α subunits. Of these only 687 variants belong to the electrophoretically demonstrable category. Accordingly, the remaing 1464 variants are left unrecongnized if electrophoresis is resorted to as a measure for hemoglobinopathy survey[14][15].

For these ten years we have devoted ourselves to the survey of abnormal hemoglobins in Japan where hemoglobinopathy is rare, and we have realized with increasing keennness for the necessity of effective methods which supplement the limitation of electrophoresis mentioned above. Thus, our attention was attracted to Dacie's heat denaturation test. We examined to simplify his technique with the least possible impairment of its quantitative character so that we might be able to use it for screening electrophoretically undetectable abnormal hemoglobins. After a series of several trials we have recently succeeded in devising two methods which are suitable for our purpose.

This paper aims to present the procedures of our heat denaturation test of hemolysate together with the results of their application to our hemoglobinopathy survey in Japan.

MATERIALS AND METHODS

Blood samples were collected from 104 normal subjects and 336 patients who visited the hospital of the Yamaguchi University School of Medicine in Ube during the period from December, 1968 to July, 1969. Several blood samples which were sent to us from other hospitals are also included as materials.

Hemolysates (Hb concentration, about 10 g/dl) were prepared from the blood samples by the conventional technique[1]. Agar gel electrophoresis (pH 8.6 and 7.0) which was reported previously[1] was employed for the detection of electrophoretically abnormal hemoglobins.

The two procedures devised for the demonstration of unstable hemoglobin in hemolysate were as follows.

(I) Screening test (centrifugation procedure)

Reagents :

1. Phosphate buffer solution (0.1 M, pH 7.4) : Dipotassium monohydrogen phosphate K_2HPO_4 (13.6 g) and monopotassium dihydrogen phosphate (2.98 g) are dissolved in distilled water in a volumetric flask (1000 ml), and made to the volume with distilled water.

2. Zinc chloride solution (0.01 M) : Zinc chloride (1.363 g) is dissolved in distilled water and made to 1000 ml with distilled water.

Procedure : Equal volumes (0.2 ml) of the hemolysate, phosphate buffer and Zinc chloride solution are mixed in a test tube. A glass capillary tube (0.3 cm in bore diameter and 12 cm in length) is inserted into the test tube. The mixture rises into the lumen of the capillary tube to a certain extent spontaneously. The test tube is tilted so that the mixture may fill the lumen of the capillary as far as 7 to 8 cm from its lower end. The capillary tube is removed from the test tube, being stoppered at its open (upper) end with a finger tip. The open end of the capillary is closed by fusing in the flame of a burner. The capillary (with its fused end downwards) is warmed at 50 °C in a water bath for 60 minutes. It is centrifuged at 3000 rpm (1700 G) for five minutes. The layer of the precipitate produced at the bottom of the capillary is read in terms of the percentage of the total column of the mixture.

Interpretation : Usually, normal hemolysates give a precipitate layer less than 3.4 % of the mixture column. Values larger than 4 per cent are suggestive of unstable hemoglobin.

(II) Spectrophotometric method (procedure for confirmation of unstable hemoglobin)

Reagents.

1. Phosphate buffer solution (0.1 M, pH 7.4) : Dipotassium monohydrogen phosphate K_2HPO_4 (13.6 g) and monopotassium dihydrogen phocphate KH_2PO_4 (2.98 g) are dissolved in distilled water in a volumetric flask (1000 ml) and made to the volume with distilled water.

Procedure : A volume of 30 μl of the hemolysate is mixed with 3.0 ml of phosphate buffer solution which was maintained at 55°C by prewarming in a test tube. The mixture is transferred into a cuvette (optical path, 1.0 cm) and measured for its absorbance at 700 mμ in a self-recording spectrophotometer with a cuvette holder maintained at 55°C continuously for 20 minutes with phosphate buffer solution as blank specimen. Normal hemolysate is treated in the same manner as described above to be used as control experiment.

Interpretaion : Absorbance larger than 0.300 at the end of 20 minutes (usually more than 2 times as high as that obtained with normal hemolysate) indicates the presence of unstable hemoglobin.

RESULTS AND DISCUSSION

The hemolysate should be clear, being free from any turbidity or precipitate before it is examined by the screening test. The amounts of precipitate obtained with normal hemolysates (104 in total) by the screening test (centrifugation method) yielded a skew curve distribution with its peak at "2.4–3.2 %". Precipitate over 4.0 % was rare. (Figure 1). Therefore, precipitate formation of 3.5 % was set at the border line level for screening test lest any sample of suspicious unstable hemoglobin should be overlooked.

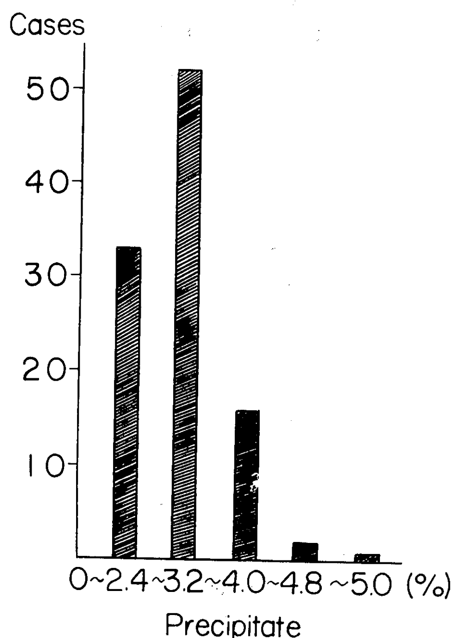

Fig. 1. Distribution of precipitate percentage in screening (centrifugation) test with hemolysates of normal subjects.

Beretta[9] was successful by application of heat denaturation (at 50 °C for 20 minutes) in the isolation and purification of Hb Torino which did not lend itself to electrophoretic analysis. Dacie[10] was also able to purify Hb Hammersmith which was inseparable by electrophoresis by warming (at 50°C for 3 hours) the mixture of the hemolysate with a buffer solution containing a small amount of zinc chloride (45 mg/dl in final concentration when mixed with hemolysate). It was necessary for us to employ zinc chloride solution (0.01 M) with phospate buffer solution (0.1 M, pH 7.4) in proportion of 1 : 1 for the purpose of obtaining a test of high sensitivity within a relatively short reaction time (60 minutes). Rotation speed of 3000 rpm (1700 G) was essential for centrifugation of the

precipitate in the capillary tube, because the reproducibility was unsatisfactory, if the tube was centrifuged more slowly.

In spectrophotometric method (for confirmation of unstable hemoglobin) the increment of absorbance per minute of the normal hemolysate-reagent mixture varied, to a certain extent, depending on temperature (53–59°C). At 53°C the increment was insufficient in sensitivity for the demonstration of unstable hemoglobin, while at the temperature range above 57°C the rise in absorbance was very rapid at first, culminating within a short time, in production of coarse particles which rendered turbidity measurment unfeasible. Therefore, 55°C was chosen as the optimum temperature of incubation. At this temperature normal hemolysates gave no significant rise in absorbance within the first 5 minutes, but showed gradual increments suitable for spectrophotometric observation thereafter until the 15th minute (Figures 2 and 3).

In Dacie's original method[13] the mixture of hemolysate and buffer solution was divided into parts. The first part (the hemolysate-buffer mixture) was allowed to stand at room temperature. The second part was warmed at 50°C for 3 hours, and centrifuged to get clear supernatant which was deprived of the particles of denatured hemoglobin. The unwarmed hemolysate-buffer solution mixture and the supernatant were measured colorimetrically for their hemoglobin concentrations by cyanmethemoglobin method. The percentage of the unstable hemoglobin to the total hemoglobin in the hemolysate was calculated by the following equation[16].

$$\text{Unstable Hb}(\%) = \frac{\text{Hb mix.} - \text{Hb sup.}}{\text{Hb mix.}} \times 100$$

Hb mix.: hemoglobin concentration of the hemolysate-buffer mixture

Hb sup.: hemoglobin concentration of the supernatant after centrifugation of the warmed hemolysate-buffer mixture.

Our spectrophotometric method aims to appraise the formation of precipitate from the unstable hemoglobin directly by the observation of increase in the turbidity (absorbance at 700 mμ) of the hemolysate-buffer solution mixture by continuous self-recording. This contributes to the simplification of the procedure.

Table 1 presents the summary of the experience with our heat denaturation tests. Six kinds of abnormal hemoglobins listed in this table were encountered in our laboratory in 1968 and 1969.

Hb Ube-1 and Hb Mie were from the patients with Heinz body anemia. Hb M Yonago and Hb M Tokyo were of Hb M diseases. The former was identical with Hb M Saskatoon ($\alpha_2\beta_2^{63Tyr}$)[17] and the latter resembled closely Hb M Hyde Park ($\alpha_2\beta_2^{92Tyr}$)[18]. Hb Ichinomiya and Hb Ohtsu were tentatively identified as Hb Gifu ($\alpha_2\beta_2^{80Lys}$)[14][19] and Hb E ($\alpha_2\beta_2^{26Lys}$)[20].

It is seen from this table that border line precipitate level of 3.5 % in centrifugation procedure will be appropriate for screening the hemolysate containing unstable hemoglobin. Hemolysate of Hb Ube-1 and Hb Mie diseases and of Hb

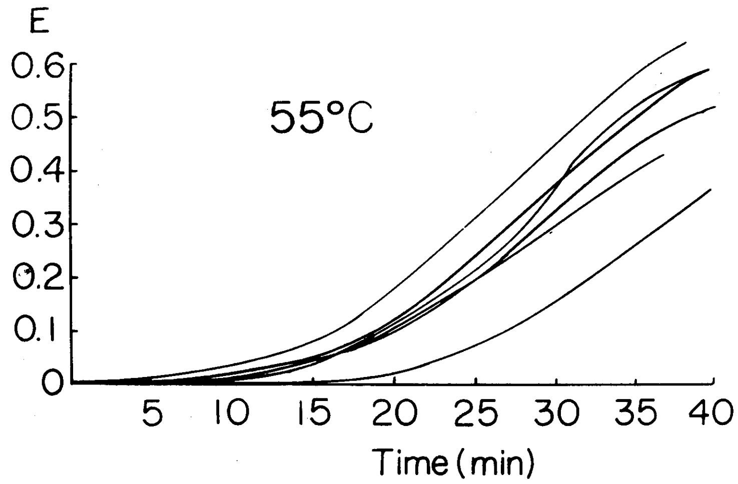

Fig. 2. Increment of absorbance of the warmed hemolysate-buffer mixture with lapse of time in normal subjects.

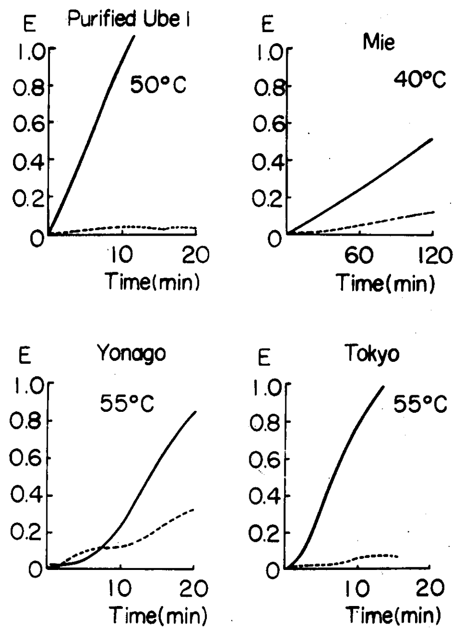

Fig. 3. Increment of absorbance of the warmed hemolysate-buffer mixture with lapse of time in some hemoglobinopathies.

Table 1. Abnormal hemoglobins as examined by our heat denaturation tests (screening procedure and spectrophotometric procedure) in 1968-1969

Hemoglobin	Electrophoresis	Screening (centifugation)	Spectrophotometric
Hb Ube-1	slow at pH 8.6	7%	(+)
Hb Mie	not demonstrable	—	(+)
Hb M Yonago	fast at pH 7.0	5%	(+)
Hb M Tokyo	fast at pH 7.0	3.8%	(+)
Hb Ichinomiya	slow at pH 8.6	3.2%	(−)
Hb Ohtsu	slow at pH 8.6	3.0%	(−)
Hb A		4.0%	(−)

(+)′: Remarkable increase in absorbance at 700 mμ (over the level two times as high as that obtained with Hb A at 20 minutes).

M Yonago and Hb M Tokyo diseases give the reading of precipitate larger than 3.5 %. Hemoglobin M's of β chain anomaly (Hb M Saskatoon and Hb M Hyde Park) have been known as heat-unstable hemoglobin. By contrast, Hb Ichinomiya and Ohtsu are as stable as Hb A in heat denaturation test. Figure 2 illustrates how rapidly the hemolysates of Hb Ube-1, Hb Mie and Hb M diseases show the distinct ascension of absorbance over the level seen in the normal hemolysate which serves as control experiment.

Interestingly, Hb Ube-1 disease exhibited many intra-erythrocytic Heinz bodies which were seen brightly glittering under fluorescence microscope. Similar fluorescence of the intraerythrocytic inclusion bodies were observed also in Hb Sabine disease by Schneider and her associates[11]. They speculated about the mechanism of the generation of Heinz bodies, saying that Hb Sabine would be highly susceptible to degeneration even while it was preserved in erythrocytes because of its loosened bonding between heme and globin. The hemoglobin thus releasing its heme away would be no longer able to maintain complete molecular conformation for want of quenching effect afforded by heme and would aggregate to form Heinz bodies which fluoresced. The globin which was prepared from Hb Sabine by Anson-Mirsky's technique fluoresced similarly. It was supposed that aromatic amino acids contained in globin would be responsible for this fluorescence.

Probably, fluorescence microscopy of the smear specimen of fresh blood may be used as a means for the demonstration of unstable hemoglobin. However, this will not be so convenient as the heat denaturation tests as a screening test, because it requires a fluorescence microscope and smear specimens which are prepared from fresh blood.

Hemoglobin Mie is worthy of special mentioning, since it is the only one of the abnormal hemoglobins encountered in our laboratory, for the detection of which heat denaturation test was particularly helpful in contrast to the uselessness of electrophoretic analysis. A brief description of Hb Mie disease will be given below.

Case Record (Hb Mie disease)[21]

A 7-year-old boy was admitted to the pediatric ward of the Mie Medical College Hospital on the 19th of February, 1968, because of cyanosis, convulsive seizures, nausea and abnormally dark colored urine.

His younger brother died of an unknown illness causing cyanosis 5 months after birth. His younger sister also was dead with similar symptoms. His mother had 3 still births in the past.

He was born as a normal mature full-term baby, weighing 2700 g at birth. The neonatal jaundice was uneventful. He had no melena, but was ill with pneumonia in his second neonatal week.

Four weeks prior to his admission to the Mie Medical College Hospital he had the onset of pyrexia accompanied by abdominal pain and frequent nausea. Three days later he had general convulsive seizure and become comatose. The unconsciousness lasted 3 days and disappeared, but pyrexia grew more evident with rashes on the skin and he passed dark brown urine. The tips of the fingers and the lips were cyanotic. Hematological examination disclosed moderate anemia with intraerythrocytic Heinz and Howell-Jolly bodies in his peripheral blood. Thus he was introduced and admitted to the Mie Medical College Hospital on the 22nd of January, 1968.

The patient was an undernourished and medium built boy who had puffy face, subicteric discoloration of the skin, and cyanosis on the lips, nail beds of the fingers and the distal portions of the extremities. The chest was clear to percussion and auscultation. The liver was palpable 3 fingers below the costal arch on the right mammillary line. The spleen was felt 1 cm below the costal arch on the left mammillary line.

The abdominal pain, nausea and dark urine were persistent after admission. These ailments could not be relieved in spite of parenteral fluid therapy. The stool became dark brown in color. There was fluctuation of the degree of cyanosis from day to day. Seven days after admission he was given a transfusion of fresh blood. This induced convulsion and unconsciousness for a while. Blood transfusions seemed to cause convulsion in this patient.

He received splenectomy on the 15th of March, but he continued to have relentless nausea, jaundice and anemia, and expired on the 29th of March.

Blood chemistry : Hemoglobin 11.9 g/dl, serum protein 8.0 g/dl, albumin/globulin 1.8, (Alb 60.8, α_1 Glb 9.5, α_2Glb 14.0, βGlb 7.4 and γGlb 8.5 per cent) icteric index 23, total bilirubin 1.2～4.8 mg/dl (direct bilirubin 0.8～1.2 mg/dl), cholinesterase 1.2 $\triangle$pH, alkaline phosphatase 4.6 Bodansky units, cholesterol

303 mg/dl, transaminase (GPT) 39 Karmen units, LDH 150 Wroblewski units, Non-protein nitrogen 23 mg/dl, urea N 12 mg/dl, sodium 134 mEq/1, potassium 3.0 mEq/1, chloride 107 mEq/1, serum iron 155 μg/dl.

Hematology: RBC 457～148×10^4/mm^3; reticulocyte 2.2−16.7 %; intraerythrocytic Heinz bodies, 8.8−31.4 % of total erythrocytes (increased to 57.8 % after splenectomy): Howell-Jolly bodies, 0.9−2.9 % (after splenectomy 2.3 %); siderocytes, 12.8 % : osmotic fragility test, normal : Dacie's autohemolysis test, normal : Coombs, tests (direct and indirect), negative.

Morphology of RBC: Moderate anisocytosis with macrocytes, normocytes and microcytes. Moderate poikilocytosis with bizarre cells, burr cells, crenated cells, ovalocytes and spherocytes. Frequent diffuse basophilia. The cells are normochromic.

WBC, 3250-11350/mm^3, with nearly normal differential count.

Thrombocyte, sufficient in number.

Intraerythrocytic enzymes: glucose-6-phosphate dehydrogenase, 7.5 units (control 7.4 u.); DPNH-diaphorase, 182 units (control 187 u.); pyruvate kinase, 16.6 units (control 10.4 u.); Reduced glutathione, 78 mg/dl (control 73 mg/dl).

Hemoglobin study: Abnormal hemoglobin was not demonstrable by agar gel electrophoresis of the patient's hemolysate. His parents and a younger sister (blood was preserved refrigerated after her death) were also negative for electrophoretically abnormal hemoglobin.

The hemolysates prepared from the patient and his younger sister gave positive result for unstable hemoglobin in heat denaturation test (centrifugation and spectrophotometric methods at 40°C. Figure 3 and 4).

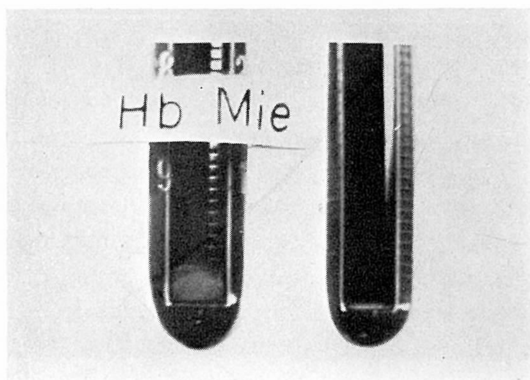

Fig. 4. Heat denaturation test (screening) of the hemolysate of Hb Mie disease in comparison with that of a normal subject. After incubating the hemolysate at 50°C for 60 minutes, the precipitate is centrifuged for 5 min. and the height of the precipitate is measured in terms of a percentage of the total volume.

Bone marrow aspiration: Erythroid hyperplasia with numerous sideroblasts.

Life span of erythrocytes: ^{51}Cr $T\frac{1}{2}$, 4 days (remarkable shortening).

Spleen: 190 g in weight; blackish brown in color. The sinuses were dilated, but phagocytosis of the reticuloendothelial cells and hemosiderin deposition were not so evident. Iron-free brownish pigment granules were seen in the cytoplasm of the cells lining the wall of the sinuses. Electron microscopy disclosed the picture of the removal of Heinz bodies from the erythrocytes by pitting function.

Urine analysis: Spectrophotometric analysis of patient's urine disclosed a shoulder-like protrusion of the absorption curve at 405 mμ. In addition there was a distinct protrusion at 345 mμ which was not seen in normal urine Gilbertsen's extraction[22] of urine for dipyrrole pigment gave substance which has a peak at 345 mμ.

SUMMARY AND CONCLUSION

Dacie's heat denaturation test was modified in order to get a practical procedure for detecting unstable hemoglobin in hemolysates. For the purpose of screening a centrifugation method was devised: hemolysate, phosphate buffer solution (0.1 M, pH 7.4), and zinc chloride solution were mixed in equal proportions (0.2 ml each) in a test tube and transferred to a capillary tube (0.3 × 12 cm). The capillary tube was sealed at one end by fusion in a flame of a burner so that the mixture might be contained safely in it, warmed at 50°C for 60 minutes, and then centrifuged at 3000 rpm (1700 G) for 5 minutes to read the amount of precipitate sedimented at the bottom in terms of the percentage of the total column of the mixture. A reading larger than 3.5 per cent is suggesive of the presence of unstable hemoglobin in hemolysate. Such a hemolysate should be examined by spectrophotometric method to confirm the result. Namely, hemolysate (30 μl) was mixed with warm (55°C) phosphate buffer solution (0.1 M, pH 7.4; 3 ml) and introduced in a cuvette. The absorbance (at 700 mμ) of the mixture was measured continuously in a self-recording spectrophotometer with constant temperature cuvette holder (55°C) for 20 minutes. Hemolysates containing unstable hemoglobin show at least an increment of absorbance two times as large as that obtained with normal hemolysates (usually the normal hemolysates gave an increase in absorbance less than 0.300 at the end of 20 minutes).

Abnormal hemoglobins seen in Ube during the period from 1968 to 1969 were examined by these tests, and an unstable hemoglobin (Hb Mie) which was unseparable from normal hemoglobin by electrophoresis was discovered. A short record of the clinical manifestation and laboratory examination of the hemolytic anemia associated with this unstable hemoglobin was presented as an example of the applcation of these methods to hemoglobin survey.

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