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Effect of temperature on the velocity of erythrocyte aggregation

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The velocity of the aggregation of human erythrocytes was examined in the range of 5–43°C with a rheoscope combined with a video camera, an image analyzer and a computer. (1) With increasing temperature, the velocity of erythrocyte aggregation induced by fibrinogen, immunoglobulin G and artificial macromolecules (dextran of 70 kDa and poly(glutamic acid) of 50 kDa) increased. However, the relationship between the velocity of erythrocyte aggregation and the temperature was different among these macromolecules. (2) In 70% autologous plasma, the velocity of erythrocyte aggregation was minimum at 15–18°C, and increased at both higher and lower temperatures. (3) The shape of erythrocyte aggregates in 12 μmol/l fibrinogen (containing 770 μmol/l albumin) and in 70% autologous plasma was dependent on temperature: three-dimensional below 15–18°C and one-dimensional (mainly rouleaux) above 15–18°C. However, the shape of aggregates in 27 μmol/l immunoglobulin G (containing 770 μmol/l albumin) was three-dimensional in all temperature ranges. (4) The temperature dependency of erythrocyte aggregation was discussed in terms of the changes of medium viscosity, of erythrocyte properties and of bridging macromolecules.

Introduction

Temperature affects the viscosity of fluid and changes the physicochemical properties of macromolecules and erythrocytes. Erythrocyte aggregation, which is induced under low shear stress (especially in the stasis of blood flow) by the macromolecular bridging between adjacent cells, must be affected by temperature. As is well known, the erythrocyte aggregation reduces the blood flow due to the increased blood viscosity and may induce the sludge in the capillary. However, the temperature dependency of the relative blood viscosity to plasma is still controversial [1]. The erythrocyte deformation in the high shear regions [2–5] and the erythrocyte aggregation in the low shear regions [6–8] must be taken into account [9]. The temperature-dependent erythrocyte sedimentation [10–12] is also closely related to erythrocyte aggregation. However, the temperature dependency of erythrocyte aggregation is not well understood. Physiologically, the erythrocyte aggregation may be important for understanding the blood flow to metabolically active tissues and to tissues exposed to the hypothermic or hyperthermic environment, in relation to the heat transfer and the oxygen and/or nutrients transport.

In the present study, we intended to investigate the effect of temperature on the velocity of erythrocyte aggregation by artificial macromolecules (dextran and poly(L-glutamic acid)) and plasma proteins (fibrinogen and immunoglobulin G), and in autologous plasma, with special reference to the shape of erythrocyte aggregates.

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Materials and Methods

Erythrocytes and plasma

Fresh blood was obtained from healthy adult males (red cell type, O^+ and A^+) and heparinized (with 10 units heparin/ml blood). Blood was centrifuged at $1200 \times g$ for 5 min at 4°C. Plasma was collected and recentrifuged at $15000 \times g$ for 20 min at 4°C to remove platelets. Erythrocytes were washed twice with about 20 vol of isotonic phosphate-buffered saline (42.6 mM Na_2HPO_4/7.4 mM NaH_2PO_4/90 mM NaCl/5 mM KCl/5.6 mM D-glucose, pH 7.4 at 25°C; 285 mosM) at 4°C, and were used within 5 h after collection of blood (during this period, the erythrocyte shape and the ATP content did not change, and the velocity of erythrocyte aggregation was not altered significantly).

Macromolecules

(i) Fibrinogen Human fibrinogen was purchased from AB Kabi (Stockholm, Sweden; grade L, 90% clottable) and was used after treating with lysine-Sepharose 4B to remove contaminated plasminogen and/or plasmin [13–15].

(ii) Immunoglobulin G (Ig G) Human IgG from Miles Lab. (Naperville, IL) was used for erythrocytes of O^+ type, because of the contamination of red cell type-specific agglutinins [16].

(iii) Polysaccharide Dextran T-70 (70.4 kDa on average) was from Pharmacia Fine Chem. (Uppsala, Sweden).

(iv) Polyamino acid. Poly-(L-glutamic acid) with the approximate molecular mass of 50 kDa (sodium salt; the degree of polymerization, 310; abbreviated as poly(Glu-50) was from Sigma Chem. Co. (St. Louis, MO).

Velocity measurement of erythrocyte aggregation

The rheoscope apparatus [17] (composed of a transparent cone-plate viscometer and an inverted microscope equipped with a temperature-controlled stage) combined with a video camera (Sony, AVC 1150, Tokyo, Japan), an image analyzer (Luzex 450, Toyo Ink Co., Tokyo, Japan) and a computer (Hewlett Packard, HP-85, Palo Alto, CA), was used for the measurement of the velocity of erythrocyte aggregation [18,19], as shown in Fig. 1.

The washed erythrocytes were resuspended in artificial medium containing bridging macromolecule (polysaccharide, poly(glutamic acid), fibrinogen or IgG) and human serum albumin (fatty acid free, fraction V from Miles Lab. Inc., Naperville,

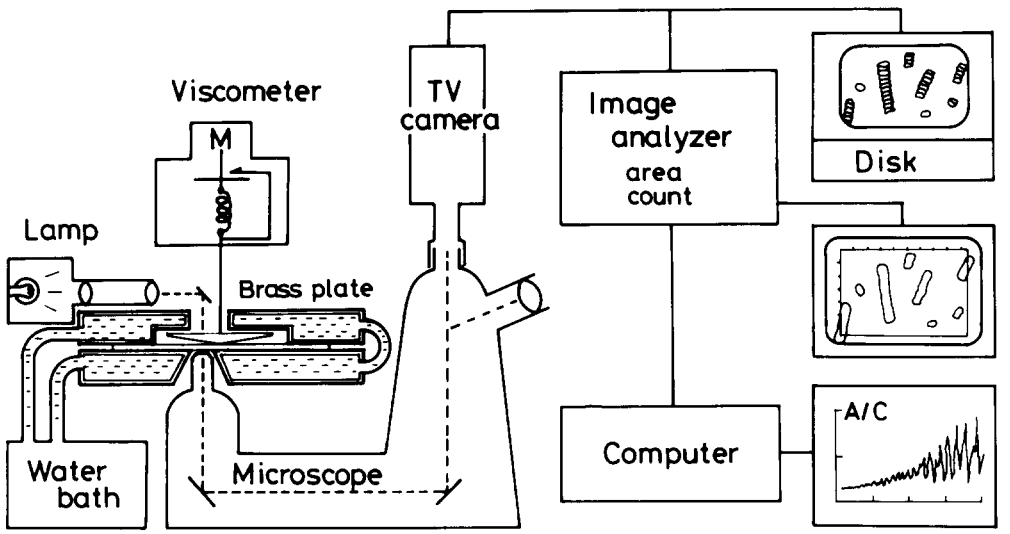

Fig. 1 Diagram of the apparatus for measuring the velocity of erythrocyte aggregation. Modified from Shiga et al. [18]. The stand of the microscope and the covering plate over the cone are made of brass, and are circulated with water of a constant temperature from a thermostatted water bath

IL; 310–770 μmol/l of albumin was used for preventing the adhesion of erythrocytes and/or aggregates on the glass plate of the rheoscope [20]) in isotonic phosphate-buffered saline (pH 7.4), or resuspended in autologous plasma (adjusted to pH 7.4; the plasma was diluted to 70% with isotonic phosphate-buffered saline, for the observation of representative rouleaux at 25 °C in our experimental condition). With respect to the sensitivity and the reproducibility of the measurement, the final hematocrit was adjusted to 0.26% (the hematocrit of the original erythrocyte suspension was determined by the microcapillary centrifugation technique). The suspension was immediately applied to the rheoscope, and the erythrocyte aggregation was observed at a constant shear rate and at a constant temperature (the gap width at the point of observation in the rheoscope was about 200 μm). The shear rate was varied by adjusting the speed of revolution of cone. Temperature was controlled by circulating water from a thermostatted bath (Lo-Temp Bath, model BL-31, Yamato Sci. Co., Tokyo, Japan) into a microscopic stage and then into a covering plate over the cone, made of brass. The count of particles (i.e., single erythrocytes, one-dimensional aggregates (rouleaux) or three-dimensional aggregates) and the total area projected by particles in a frame of the video image (actual frame size, 200 × 150 μm^2) were consecutively encoded by the analyzer at an interval of approximately 1.3 s, and transfered to the computer. The velocity of erythrocyte aggregation was expressed by the increment of area/count per unit time (v, μm^2/min) [14–16,18,19,21].

Measurement of viscosity of media

The viscosity of various media containing macromolecules was measured at various temperatures by a cone-plate viscometer (model E, mounted 0.8° cone; Tokyo Keiki Co., Tokyo, Japan), for the correspondence to the rheoscope measuring the velocity of erythrocyte aggregation.

Morphological examinations

The shape of erythrocytes, rouleaux and aggregates in the process of erythrocyte aggregation was observed under the inverted microscope of rheoscope directly. The erythrocyte aggregates formed under a shear rate of 7.5 s^{-1} were photographed 10 min later, since the erythrocyte suspension was applied to the rheoscope.

The shape of erythrocytes at various temperatures was observed with a scanning electron microscope (Hitachi, S-500A, Japan), after fixing in the isotonic medium containing 1% glutaraldehyde at the same temperature as the experimental conditions for 16 h and further in 1% OsO_4.

Results

Erythrocyte aggregation by artificial macromolecules

Many artificial macromolecules, such as polysaccharides and polyamino acids [9,21,22], aggregate erythrocytes. Fig. 2a shows the temperature dependency of erythrocyte aggregation induced by 290 μmol/l Dextran T-70 and 80 μmol/l poly(Glu)-50 in isotonic phosphate-buffered saline

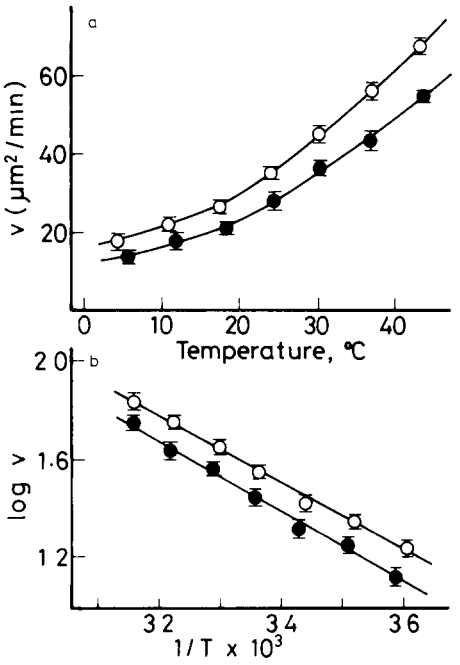

Fig 2 Temperature dependency of the velocity of artificial macromolecules-induced erythrocyte aggregation (a) Erythrocyte aggregation induced by 290 μmol/l Dextran T-70 (●) and 80 μmol/l poly(Glu)-50 (○) in the presence of 310 μmol/l albumin Measured in isotonic phosphate-buffered saline, pH 7.4 (at 25 °C), at a shear rate of 7.5 s^{-1} (b) Plot of log (velocity) vs $1/T$ (K). Symbols as in the (a).

containing 310 μmol/l albumin. With increasing temperature of the medium, the velocity of erythrocyte aggregation induced by Dextran T-70 and poly(Glu)-50 increased: the velocity of erythrocyte aggregation by 80 μmol/l poly(Glu)-50 is larger than that by 290 μmol/l Dextran T-70 in all temperature ranges examined here. The plot of log (velocity) vs. $1/T$ (K) gave a straight line for both macromolecules, as shown in Fig. 2b.

Erythrocyte aggregation by fibrinogen

Fibrinogen aggregates erythrocytes very effectively [9,14,15,23–26]. The temperature-dependent erythrocyte aggregation induced by 8.8 and 12 μmol/l fibrinogen (containing 770 μmol/l albumin) is shown in Fig. 3a. With increasing temperature, the fibrinogen-induced erythrocyte aggregation was accelerated, but the velocity change was small between 15 and 30 °C. In the case of fibrinogen-induced erythrocyte aggregation, the plot of log (velocity) vs. $1/T$ (K) did not give a straight line, as shown in Fig. 3b.

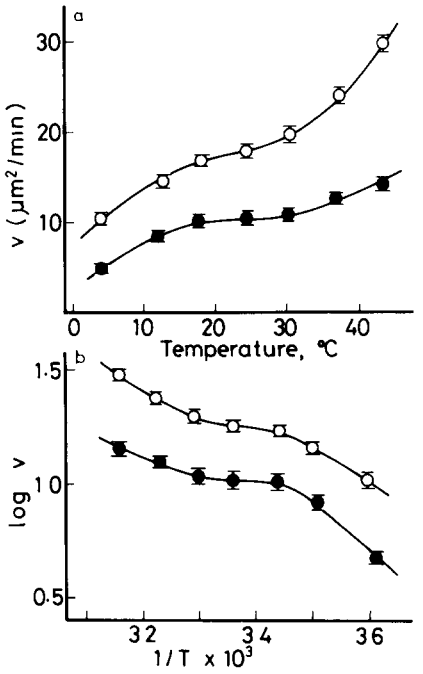

Fig 3 Temperature dependency of the velocity of fibrinogen-induced erythrocyte aggregation (a) Erythrocyte aggregation induced by 8 8 μmol/l (●) and 12 μmol/l (○) fibrinogen in the presence of 770 μmol/l albumin Conditions as in Fig. 2 (b) Plot of log (velocity) vs $1/T$ (K) Symbols as in the (a)

Erythrocyte aggregation by IgG

Pathophysiologically, IgG is an important macromolecule for inducing erythrocyte aggregation [9,15,27]. The temperature-dependent erythrocyte aggregation induced by 27 μmol/l IgG (containing 770 μmol/l albumin) is shown in Fig. 4a. With increasing temperature, the velocity of erythrocyte aggregation increased sigmoidally and above 30 °C, the velocity began to saturate. The pattern of the temperature dependency of velocity was different between IgG- and fibrinogen-induced erythrocyte aggregation. The plot of log (velocity) vs. $1/T$ (K) did not give a straight line, as shown in Fig. 4b.

Erythrocyte aggregation in plasma

The temperature dependency of erythrocyte aggregation in 70% autologous plasma is shown in Fig. 5. The relation between the velocity of erythrocyte aggregation and the temperature was quite different from those observed for other macromolecules. (1) Above 18 °C, the velocity of erythrocyte aggregation increased, with increasing

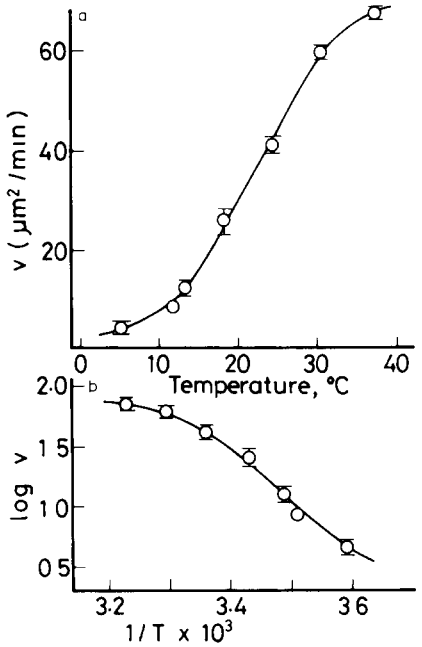

Fig. 4 Temperature dependency of the velocity of IgG-induced erythrocyte aggregation (a) Erythrocyte aggregation induced by 27 μmol/l IgG in the presence of 770 μmol/l albumin Conditions as in Fig 2 (b) Plot of log (velocity) vs $1/T$ (K)

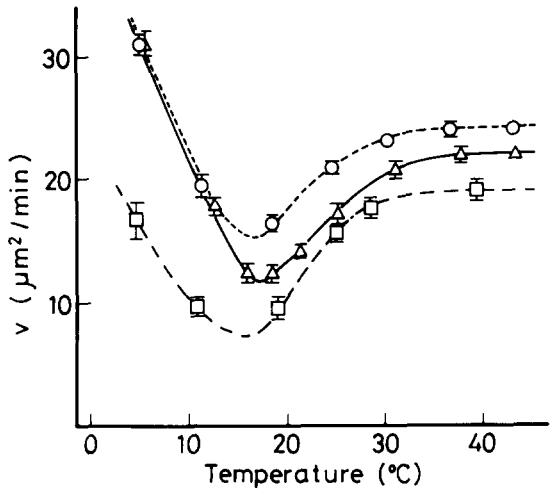

Fig. 5 Temperature dependency of the velocity of erythrocyte aggregation in autologous plasma Measured in 70% autologous plasma + 30% isotonic phosphate-buffered saline (pH 7.4, at 25 °C) at a shear rate of 7 5 s^{-1} Samples NM (○, red cell type A^+), KK (□, red cell type A^+), MS (△, red cell type 0^+)

temperature, and the velocity was nearly constant above 30 °C. (ii) Below 16 °C, the velocity increased, with decreasing temperature. (iii) The velocity was minimum at 16–18 °C. (iv) The temperature dependency of erythrocyte aggregation was similar among individuals, but the degree of erythrocyte aggregation was different probably due to the differences in composition of plasma proteins, in rheological properties of individual erythrocytes and in the interaction between plasma proteins and erythrocytes.

Morphological characteristics of erythrocyte aggregates

Rheologically, the shape of erythrocyte aggregates, especially induced by plasma proteins, is of great interest. The erythrocyte aggregates formed under various temperatures at a shear rate of 7.5 s^{-1} were photographed 10 min after the erythro-

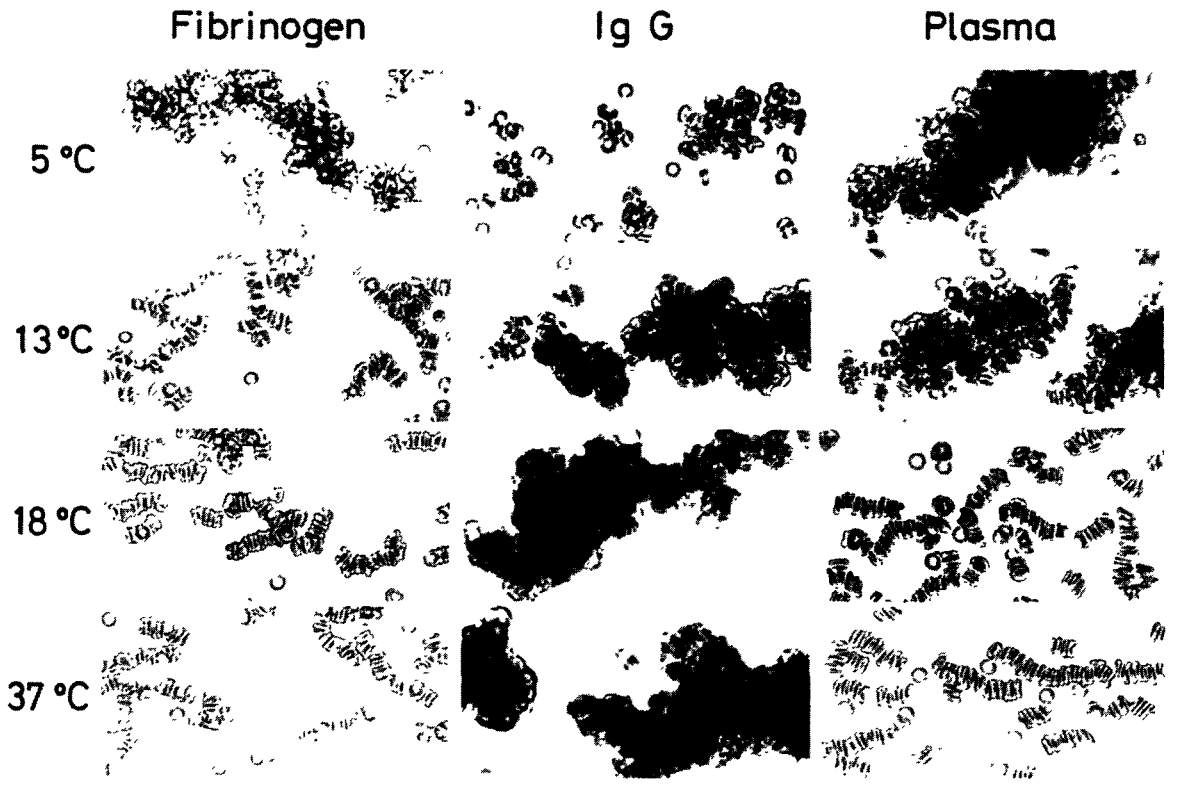

Fig 6 Microphotographs of aggregated erythrocytes The erythrocyte aggregation was induced in 12 μmol/l fibrinogen + 770 μmol/l albumin (fibrinogen), in 27 μmol/l IgG + 770 μmol/l albumin (IgG) and in 70% autologous plasma + 30% isotonic phosphate-buffered saline (plasma) at a shear rate of 7.5 s^{-1} at 5, 13, 18 and 37 °C 10 min later, the erythrocyte aggregates were photographed

cyte suspension was applied to the rheoscope. The results are summarized in Fig. 6.

In 70% autologous plasma, the erythrocyte aggregates formed at high temperature were distinctly different from those formed at low temperature: i.e., the erythrocyte aggregates above 18° C were one-dimensional (linearly arranged) rouleaux, while those below 16° C were three-dimensional (complicatedly piled and branched) aggregates. The shape of erythrocyte aggregates was continuously altered around 16–18° C.

The fibrinogen-induced aggregates below 13° C were clearly three-dimensional, while the aggregates above 18° C were mostly rouleau-like. The shape change was also continuous between 13 and 18° C. The shape of fibrinogen-induced aggregates was similar to that in 70% autologous plasma in all temperature ranges in the present experimental condition.

On the other hand, the temperature-dependent shape change of IgG-induced erythrocyte aggregates was not observed. Finally, the aggregates were three-dimensional in all temperature ranges.

Discussion

Various factors for temperature-dependent erythrocyte aggregation

The temperature dependency of erythrocyte aggregation in plasma has been observed by the erythrocyte sedimentation [6,10–12,28], by the echogenicity of erythrocyte aggregates [7] and by the photometric aggregometry [8]. However, the mechanism of temperature-dependent erythrocyte aggregation is not fully understood.

On the dynamics of erythrocyte aggregation, Chien and Jan [29] have speculated as follows: when two erythrocytes are close, the free end of macromolecule already adsorbed on one cell adheres onto the other cell, if the distance between cells is adequate to minimize the electrical repulsion between negatively charged erythrocyte surfaces. Such bridge formation over a limited area brings the adjacent surface into sufficiently close range for further bridging to proceed. The flexibility of erythrocyte membrane facilitates the progression of such bridge formation and results

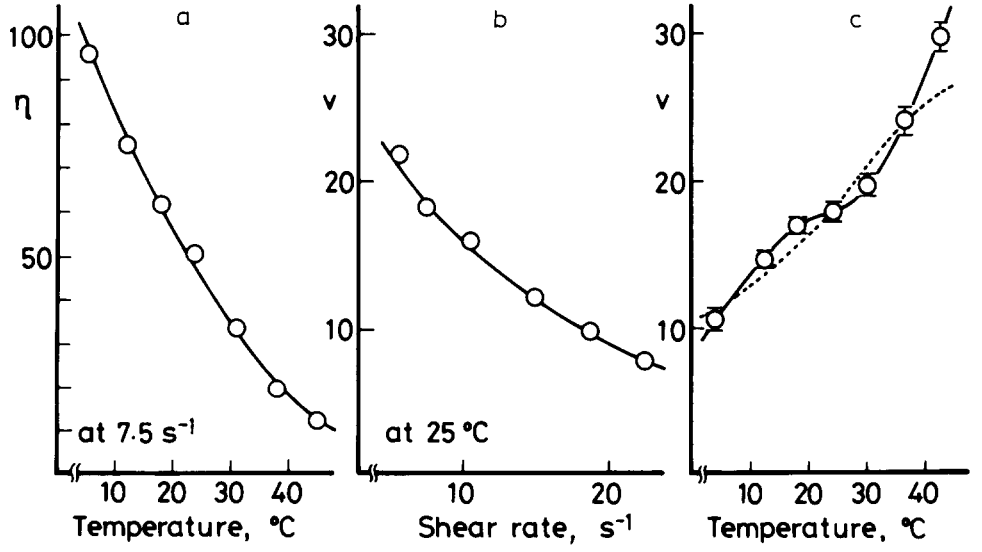

Fig 7 Erythrocyte aggregation in 12 μmol/l fibrinogen containing 770 μmol/l albumin (a) Temperature dependency of the medium viscosity (η, cP) measured at a shear rate of 7.5 s^{-1} (b) Shear rate dependency of the velocity of erythrocyte aggregation (v, μm^2/min), measured at 25° C (c) Temperature dependency of the velocity of erythrocyte aggregation (v, μm^2/min) O——O, observed, — — —, estimated from the viscosity change by temperature, as described in the text

in a maximum area of cell surface bridges by macromolecules. The present experiment was carried out under a constant concentration of erythrocytes (0.26% in hematocrit) and macromolecules. Therefore, the effect of temperature on the velocity of erythrocyte aggregation is discussed in terms of (1) the collision frequency among erythrocytes, (2) the mechanical shearing force, (3) the conformation of bridging macromolecules, (4) the properties of erythrocytes, especially the deformability and the shape, and (5) the pH of medium.

(1) Collision frequency. The frequency of collision among erythrocytes is essentially important for inducing erythrocyte aggregation. According to Smoluchowski [30], contrary to Brownian motion, the collision frequency among particles in a laminar shear flow is independent of the viscosity of the medium and/or temperature. Under a constant shear rate (7.5 s^{-1}, in our experiment), the collision frequency among erythrocytes may be independent of temperature.

(2) Mechanical shearing force. Since the viscosity of medium is increased by lowering temperature, the mechanical shearing force (i.e., the product of the viscosity of medium and the shear rate) increases at a constant shear rate. Thus, the aggregated erythrocytes are dispersed and/or the erythrocyte aggregation is inhibited [15,18,21]. In order to understand the effect of temperature on the mechanical shearing force, (i) the temperature dependency of viscosity of medium at a constant shear rate (7.5 s^{-1}) and (ii) the shear rate dependency of the velocity of erythrocyte aggregation at a constant temperature (25 °C) were examined for fibrinogen-induced erythrocyte aggregation, as shown in Fig. 7. With increasing temperature, the viscosity of 12 μmol/l fibrinogen solution (containing 770 μmol/l albumin) decreased (Fig. 7a). And, with increasing the shear rate, the velocity of erythrocyte aggregation decreased (Fig. 7b).

From the view point of the shear stress, the increase of viscosity is equivalent to the increase of the shear rate, and vice versa; namely the velocity of erythrocyte aggregation at t °C may be same as that at the shear rate of $\dot{\gamma}_t \cdot \eta_t / \eta_{25}$ ($\dot{\gamma}_t$, shear rate (7.5 s^{-1}) at t °C; η_t and η_{25}, viscosity of medium at t °C and 25 °C, respectively) at 25 °C (temperature standardized for this trial).

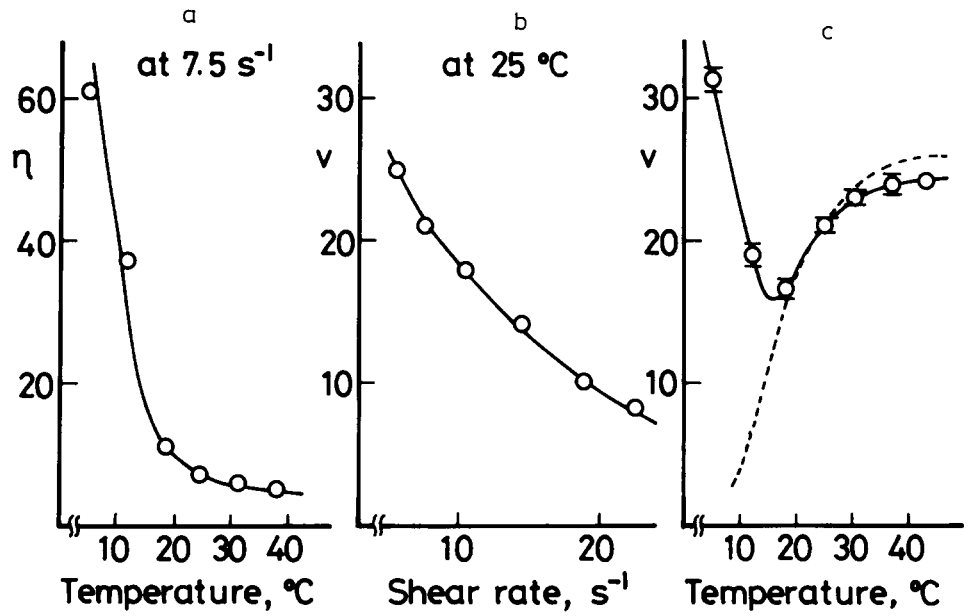

Fig. 8. Erythrocyte aggregation in 70% autologous plasma + 30% isotonic phosphate-buffered saline (a) Temperature dependency of the medium viscosity (η, cP) measured at a shear rate of 7.5 s^{-1} (b) Shear rate dependency of the velocity of erythrocyte aggregation (v, μm^2/min), measured at 25 °C (c) Temperature dependency of the velocity of erythrocyte aggregation (v, μm^2/min) O——O, observed, — — —, estimated from the viscosity change by temperature, as described in the text

The velocity of erythrocyte aggregation estimated from the medium viscosity and the shear rate is shown by dotted line in Fig. 7c. The estimated velocity is similar to the observed velocity.

The velocity of erythrocyte aggregation in 70% autologous plasma was also estimated, based on the change of shear stress by temperature. As shown in Fig. 8, the estimated velocity was similar to the one observed above 18°C, but was definitely different below 18°C. Recently, Neumann et al. [8] have shown that the overall process of erythrocyte aggregation is retarded with decreasing temperature, due to the increased plasma viscosity. The difference from our results must be largely due to the methodological differences, such as in the collision frequency (including the shear rate), the erythrocyte and plasma concentrations, the shape of erythrocyte aggregates and so on. In any way, our results mean that the temperature dependency of erythrocyte aggregation in plasma cannot be explained solely by the change of viscosity, namely the change of shear stress.

(3) Properties of macromolecules The interaction between macromolecule and erythrocyte is important for the erythrocyte aggregation, though the binding of macromolecules to the erythrocyte surface does not necessarily bridge between erythrocytes. Rampling [31] has shown that the binding of fibrinogen is not affected by temperature between 4 and 37°C. On the other hand, Neumann et al. [8] have recently observed the increase in adsorptive energy of erythrocyte aggregation with decreasing temperature.

Generally, among the same molecular species, a macromolecule with a higher molecular weight is stronger for inducing the erythrocyte aggregation than that with a lower molecular weight [9,21]. However, poly(glutamic acid) (80 μmol/l) has a stronger ability to form the erythrocyte aggregates than dextran (290 μmol/l) in the temperature range of 5–43°C (Fig. 2), in spite of the fact that the molecular weight of poly(Glu)-50 is lower than Dextran T-70. Furthermore, we have suggested that the interacting mode of poly(glutamic acid) to the erythrocyte surface is different from that of dextran [21] Therefore, the conformation of macromolecules [32–34] and the interaction with the erythrocyte surface [21] are important for bridging among erythrocytes. This may be reflected in the different patterns in the temperature dependency of the velocity of erythrocyte aggregation (Figs. 2, 3 and 4).

As clarified in the present experiment, the shape of erythrocyte aggregates depends on the molecular species of bridging macromolecules. Especially above 15–18°C, the fibrinogen-induced erythrocyte aggregates were one-dimensional (rouleaux), while the IgG-induced ones were three-dimensional, though they were three-dimensional for both proteins below 15–18°C. Shiga et al. [19] have observed that the three-dimensional aggregates are not formed in low concentration of γ-globulin In addition to the difference of molecular species, the reversible conformational change of fibrinogen and IgG at the transitional temperatures of 11–16°C [35] and of 25–35°C [36], respectively, may partly contribute to the characteristic temperature dependency of erythrocyte aggregation. The temperature dependency of erythrocyte aggregation in 70% autologous plasma was quite different from that by fibrinogen or IgG, although the shape of erythrocyte aggregates in 70% autologous plasma was similar to that of fibrinogen-induced ones in all temperature ranges The characteristics of erythrocyte aggregation in autologous plasma must be due to the complex composition of plasma proteins. The relative proportion of fibrinogen and γ-globulin may be an important factor for the interpretation of erythrocyte aggregation in autologous plasma.

(4) Properties of erythrocytes The shape, especially the ratio of surface area to volume, is important for the erythrocyte aggregation by macromolecular bridging. The erythrocyte shape at various temperatures was observed by scanning electron microscopy (Fig. 9), and the diameter and the maximum thickness of cells were measured on the photographs. At high temperature the cells were clearly flattened. compared with the cells at 20°C, the cells at 2°C decreased 9% in diameter ($P < 0.001$, by Student's t-test) and increased 20% in thickness ($P < 0.001$), while those at 37°C increased slightly ($< 1\%$) in diameter and decreased 4% in thickness (statistically not significant). $\pi \times (\text{radius})^2 \times (\text{thickness})$, a rough measure of cell volume, was not altered by temperature. Murphy [37] has reported that, with increasing temperature, the diameter of the cell increased with main-

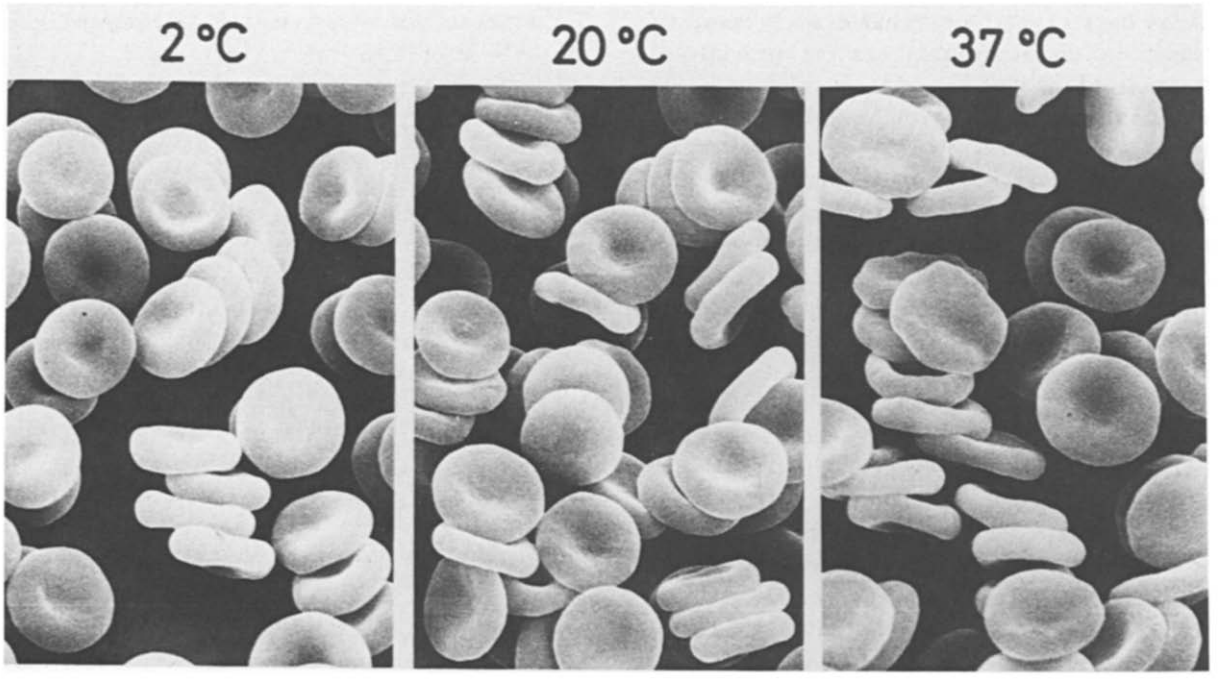

Fig 9 Temperature-dependent shape change of erythrocytes Observed by scanning electron microscopy, after fixing in 1% glutaraldehyde for 16 h and then 1% OsO_4

taining a constant cell volume. The flattening of erythrocytes may partly explain the accelerated erythrocyte aggregation at high temperature.

The deformability of erythrocyte is important for the facilitation of erythrocyte aggregation, as described above. We have observed that the cross-linking of membrane proteins with diamide inhibits the erythrocyte aggregation [38]. Williamson et al. [2] and Hanss and Koutsouris [3] have observed the increased deformability of erythrocytes at high temperatures and the thermal transition of deformability around 20 °C. The thermal transition on the fluidity of membrane lipids [3,39] and on the mobility of membrane proteins [40] may be related to the alteration of erythrocyte deformability and then may affect the change of erythrocyte aggregation in both the velocity and the shape around 15 °C.

The change of internal viscosity of erythrocytes by temperature may affect the erythrocyte aggregation through the modification of erythrocyte deformability. The transition temperature has not been found for a hemoglobin solution of 30 g/dl (4.7 μmol/l) [3]. In addition, the effect of temperature on the rheological behavior of erythrocytes may be different among individuals and may vary with the composition of the plasma.

We suppose that in the fibrinogen-induced erythrocyte aggregation, the erythrocytes at high temperatures easily form one-dimensional rouleaux due to the increased deformability so as to get the maximum area of cell surface bridged by fibrinogen, while those at low temperatures form complicatedly piled and branched aggregates due to the decreased deformability (and the increased adsorptive energy [8]) which prevent the transition from face-to-rim to face-to-face adhesion.

(4) pH of medium. The erythrocyte aggregation is affected by the pH of medium [18]: the velocity of erythrocyte aggregation in autologous plasma increases in alkaline pH, while it decreases in acidic pH. With increasing temperature from 4 to 44 °C, pH of the medium decreased 0.06 pH units (in the present experimental condition). The lowering of 0.06 pH units decreases only 0.5 μm^2/min in the velocity of erythrocyte aggregation [18], which is within the experimental error.

Finally, the temperature dependency of eryth-

rocyte aggregation is concluded as follows: the velocity of erythrocyte aggregation is mainly influenced by (a) the change of shear stress due to the altered viscosity of medium, (b) the changes of morphological and viscoelastic properties of erythrocytes and (c) the structural change of bridging macromolecules. On the other hand, the shape of erythrocyte aggregates is mainly affected by the properties and/or the composition of bridging macromolecules: fibrinogen prefers to form one-dimensional rouleaux, while IgG forms three-dimensional aggregates.

Pathophysiological implication of temperature-dependent erythrocyte aggregates

The erythrocyte aggregation is an important factor for the blood flow. Especially in the stasis of blood flow, the erythrocyte aggregation is accelerated. The temperature-dependent change of erythrocyte aggregation in plasma must be taken into account for an understanding of the microcirculation in hypothermic tissues (e.g., skin exposed to cold environment) or hyperthermic tissues (e.g., metabolically active tissues).

As shown in the present experiment, the decreased velocity of erythrocyte aggregation with decreasing temperature from 30 °C to 16–18 °C is accommodative for the maintenance of blood flow. On the other hand, the saturated velocity of erythrocyte aggregation above 30 °C is preferable for the blood supply to metabolically active tissues and the heat transfer from such tissues to whole body.

However, the increased velocity of erythrocyte aggregation below 16–18 °C may induce circulatory disturbances.

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