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THE EFFECT OF MEMBRANE-STRAIN RATE AND OF TEMPERATURE ON ERYTHROCYTE FRAGILITY AND CRITICAL HEMOLYTIC VOLUME

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SUMMARY

1. A new and accurate method is described for carrying out osmotic hemolysis at different rates. The method requires a variable-speed syringe pump. The NaCl concentration, the relative tonicity, T, and the index of the membrane-straining rate, $(-1/T^2)\cdot(dT/dt)$, can be precisely known for any time point during hemolysis.

2. The critical hemolytic volume, V_c, obtained by the GUEST AND WING method, was found to be independent of the membrane-strain rate and independent of the temperature. This finding is consistent with the hypothesis of RAND that the membrane hemolyzes at the same strain.

3. The osmotic fragility was reduced at higher temperatures and at slower hemolyzing rates. Since V_c was independent of temperature and strain rate, a shift in osmotic fragility did not necessarily mean that the V_c was larger.

4. The prelytic release of K^+ in fast hypotonic hemolysis amounted to 10% of the intracellular K^+. This increased to about 20% for slow hemolysis. A prelytic loss of intracellular K^+ quantitatively explains the reduced osmotic fragility observed in gradual hemolysis; a prelytic loss of K^+ most probably also accounts for the reduced fragility at elevated temperatures.

5. The time course of the changes in mean cell volume that a population of erythrocytes undergo in osmotic hemolysis to become spherical ghosts was monitored by a Coulter counter and computer. In 0.30% NaCl the mean cell volume became 120–125 μ^3 within 10 sec, then remained unchanged for 30 sec (or sometimes dropped transiently by 5 μ^3), and the mean cell volume of the spherical ghosts reached a final value of about 152 μ^3. These results are compatible with the idea that individual erythrocytes hemolyze at approximately the same V_c (of about 150 μ^3) but at different times (so that the critical mean cell volume is less than the V_c).

6. The volumes of cells in isotonic solution were unchanged over a wide range of temperature. Considering the observations that the GUEST AND WING V_c was independent of temperature and the HOFFMAN V_s (sealing volume) varied markedly with temperature, the facts indicate either that (a) there was a transient collapse of the ghost before sealing, or (b) membrane sealing occurred before transmembrane equilibration of hemoglobin. The sealing volume, V_s, does not necessarily, therefore, have the same value as V_c.

INTRODUCTION

It is known that hypotonic hemolysis carried out rapidly occurs at a higher salt concentration than gradual or slow hemolysis[1,2]. This observation has been interpreted by KATCHALSKY *et al.*[1] as suggesting that when the erythrocyte swells in hypotonic solution at a gradual rate it undergoes more swelling before hemolysis occurs. It was observed by RAND[3,4], however, using micropipettes, that hemolysis occurred at a critical amount of membrane stretch or strain, rather than stress. According to the viscoelastic membrane model proposed by KATCHALSKY *et al.*[1], therefore, the V_c (or the critical hemolytic volume immediately before hemoglobin release) obtained under conditions of gradual hemolysis should be higher than the V_c obtained in fast hemolysis. On the other hand, according to the findings of RAND[3,4], the V_c values in fast and slow hemolysis should be the same.

A marked reduction in osmotic fragility is also brought about by an elevation in temperature[5–10]. Such a reduction in fragility might be obtained if the V_c were increased at higher temperature.

The experiments described in the present paper were undertaken to obtain and compare the V_c values in fast and slow hemolysis and in fast hemolysis at different temperatures.

MATERIALS AND METHODS

Procedure for determining microhematocrits of 30 % cell suspension

Human blood, drawn from a fasting volunteer, was heparinized at 75 units/ml blood, centrifuged for 15 min at 1500 × *g* and the plasma and buffy coat were aspirated. Separate experiments had indicated that the amount of heparin added had no effect on the osmotic fragility. The cells were then washed once by resuspending in 154 mM NaCl in 10 mM sodium phosphate buffer (pH 7.0). After centrifugation and aspiration of the supernatant, 0.1-ml aliquots of the packed erythrocytes were pipetted into 12 mm × 75 mm glass test tubes. As soon as possible after this step, 0.2-ml aliquots of hypotonic NaCl solutions, buffered at pH 7 by 10 mM sodium phosphate, were added to the tubes. The contents were immediately mixed thoroughly by "buzzing" the tube against a vortex mixer or whirlimixer.

Small aliquots of the final erythrocyte suspension were drawn up into microhematocrit tubes (MSE, Measuring and Scientific Equipment Co., Great Britain), approx. 0.8 mm internal diameter and 75 mm long. For drawing up the blood, it was very convenient to insert the microhematocrit tube into the bulb assembly of a Drummond Scientific (U.S.A.) micropipette. One dry end of the microhematocrit tube was sealed with Clay–Adams (New York) Seal-Ease plastic clay and the tube placed horizontally in a Hawksley (Great Britain) centrifuge. The tubes were centrifuged at 7000 × *g* for one or two or occasionally three periods of 8 min each until the hematocrit value was constant and until complete translucency of the packed cell column occurred (KOEPPE's[11] criterion). The heights of the total and packed cell columns were read merely by placing the tube adjacent to a transparent millimeter rule against the sunlight; since the tubes were very thin, parallax errors were minimal, and reproducibility was within 2 %. The height of the packed cell volume was corrected for the trapped extracellular fluid, which amounts to about 2 % at 6000 × *g* (see ref. 12).

The hematocrit of the packed erythrocytes immediately after washing in 154 mM NaCl, 10 mM sodium phosphate buffer (pH 7), was usually between 90 and 92 %; therefore, the final hematocrit value in isotonic saline was approx. 30 %.

The microhematocrits determined at low and high temperatures were done by placing the test tube containing the erythrocyte aliquot into either an ice–water bath or a water bath at 37° before adding the cold or warm hypotonic solution. The microhematocrit tubes were centrifuged either in a refrigerator at 4° or a dry oven incubator at 37°, both the centrifuge and the centrifuge rotor head being separately prechilled or preheated. The rotor of the Hawksley centrifuge warmed up by approx. 2–3° for each period of centrifugation. Before recentrifugation a second or third time the rotor temperature was brought back to the desired value by placing it in contact for 2 min with a prechilled or preheated metal slab.

Procedure for determining hematocrits of 0.3–0.5 % cell suspensions

For dilute erythrocyte suspensions, 0.3–0.5 % (v/v), hematocrits were obtained by the method of GUEST AND WING[13] employing regular Van Allen tubes. For an accurate value of the total volume in each Van Allen tube, the tube together with its spring clip was weighed on a Stanton CL5D balance to within 0.1 mg before and after the cell suspension was sucked up into it. The tubes were centrifuged for 15 min at 1600 × *g* in an International clinical model centrifuge. Such short centrifugation times at low centrifugal force do not result in complete packing or in KOEPPE's[11] criterion and approx. 6–7 % of the extracellular fluid remains trapped[12,14]. The packed cell volumes were corrected for this trapping. Longer centrifugation times were avoided because of the possibility of warming the tubes in the centrifuge.

Pump method for carrying out hypotonic hemolysis slowly

The method of gradual or slow hemolysis introduced by DANON *et al.*[2] employs a dialysis bag to retard by diffusion the rate of fall of NaCl concentration in contact with the erythrocytes. The exact concentration of NaCl inside the bag containing the cells cannot be determined easily without inventing complicated sampling micromethods. Results of dialysis bag experiments also have a lower reproducibility than fast hemolysis experiments, as pointed out by KATCHALSKY *et al.*[1].

The method of DANON *et al.*[2], therefore, was modified and improved as follows. An 1-ml aliquot of a 2.4 % suspension of cells in 154 mM NaCl in 10 mM sodium phosphate buffer (pH 7) was weighed out into a glass liquid scintillation vial (24 ml capacity). A preweighed magnetic micro stirring bar was placed inside and the vial placed on a magnetic stirring platform. The cells were then hemolyzed slowly by adding 5 ml of a hypotonic NaCl solution (always containing 10 mM sodium phosphate buffer, pH 7) from a syringe in a Harvard constant-infusion pump. The output from the syringe pump was fed to the erythrocyte suspension by a thin flexible polyethylene tube which touched the side of the glass vial several mm above the meniscus. The exact total amount of fluid delivered was obtained by weighing the vial and contents after the end of the pump infusion period. The cells were stirred constantly and the vial was routinely transferred half-way through the infusion to another magnetic stirring platform in order to maintain the vial at constant room temperature.

The concentration of NaCl inside the vial can be accurately calculated for any time point during the pump infusion by means of the following equation:

$$c_t = \frac{c_i V_i + c_p \cdot \frac{dQ}{dt} \cdot t}{V_i + \frac{dQ}{dt} \cdot t} \quad (1)$$

where c_t is the NaCl concentration in the vial at time, t; c_i is the starting NaCl in the vial (= 154 mM NaCl); V_i is the weighed starting volume in the vial; c_p is the NaCl concentration in the syringe, dQ/dt is the infusion rate in ml/min, and t is the time elapsed since the start of infusion.

The relative tonicity, T, at time t, will be c_t/c_i, consistent with PONDER's terminology[15].

Hemoglobin determinations

Hemoglobin was determined from the absorbance of the solution at 543 nm in a Beckman DB grating spectrophotometer or by the micro benzidine method[16,17].

Potassium determinations

The lytic and prelytic release of K^+ in fast and slow hemolysis was measured using a Hewlett–Packard atomic absorption flame spectrophotometer.

Determination of the time course of the erythrocyte volume changes occurring in osmotic hemolysis

A stock erythrocyte suspension of $1.8 \cdot 10^6$ cells/ml in 154 mM NaCl (pH 7) in 10 mM sodium phosphate buffer was prepared. Aliquots of 0.5 ml were then added to Coulter counter plastic vials containing 10 ml of 51 mM NaCl in 10 mM sodium phosphate buffer (pH 7). The cells were then immediately counted and sized using a Coulter counter Model F, and a mean cell volume computer (Coulter Electronics, U.S.A.).

The aperture diameter was 100 μ, the aperture setting 16, and the attenuation 0.707. Mean cell volume readings from the Coulter computer were taken four times during the course of osmotic hemolysis in each vial tested, an aliquot of 0.5 ml passing through the Coulter counter for each determination. Approx. 20000 cells are averaged by the mean cell volume computer, while about 40000 cells are counted altogether. Replicate readings of the mean cell volume meter had a coefficient of variation of less than 1 % in agreement with the findings of HATTERSLEY AND RAGUSA[18].

RESULTS

Effect of temperature on V_c

The effect of temperature on the critical hemolytic volume, V_0, was studied using microhematocrits and the GUEST AND WING method[13]. The results in Fig. 1 show that while temperature had a marked effect on the osmotic fragility, there was very little effect on the maximum value of the microhematocrit attained in hypotonic solutions. Temperature also had virtually no effect on the volumes of cells in isotonic and slightly hypotonic media, in agreement with the findings of MURPHY[10].

It is more convenient to express the hematocrit values as relative cell volumes, V/V_0, where V_0 is the cell volume or hematocrit of the erythrocytes in 154 mM NaCl, 10 mM sodium phosphate buffer (pH 7). This has been done in Fig. 2A for the data of

the typical experiment in Fig. 1. Using the GUEST AND WING method[13] for correcting for the associated percent hemolysis, the corrected relative cell volumes are plotted in Fig. 2B.

The results in Fig. 2B demonstrate that V_c, the corrected critical hemolytic volume, had approximately the same value of 1.78 at all three temperatures of 5, 20 and 35°. A number of determinations of V_c at different temperatures were carried out and these are presented in Fig. 3. Also plotted in Fig. 3 are the results of HOFFMAN *et al.*[19] wherein the sealing volume, V_s, of human ghosts was determined by measuring the amount of hemoglobin trapped by the hemolyzed cells. It is seen that the

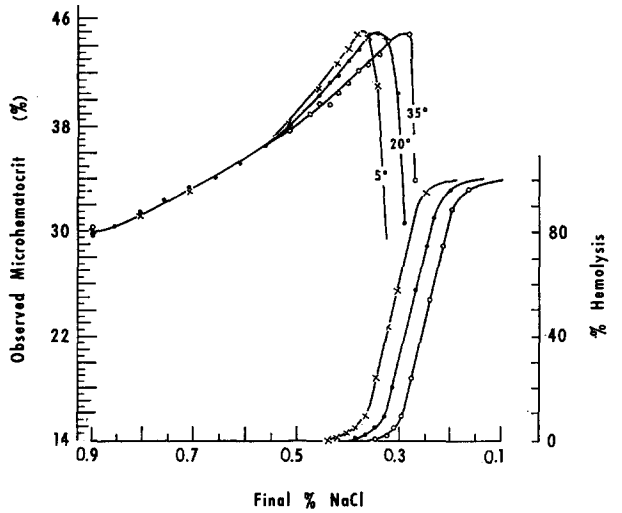

Fig. 1. Relationship of hemolytic volume and temperature, showing that the maximum microhematocrit value obtained in a series of hypotonic solutions is independent of the temperature. The osmotic fragility is markedly reduced, however, at higher temperature. Note that the microhematocrit volumes in isotonic solution are also unaffected by temperature.

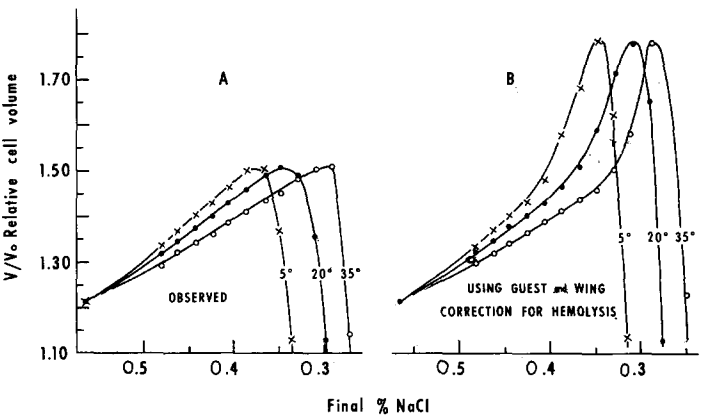

Fig. 2. Relationship of critical hemolytic volume and temperature, showing that the V_c is independent of the temperature. The ordinate, V/V_0, represents the relative cell volume, where V_0 is the value for the microhematocrit in 0.9% NaCl and V is the microhematocrit in hypotonic solution. (A) The observed data where V_c is about 1.52 at all three temperatures. (B) The result of correcting the data of A using the GUEST AND WING correction method and the % hemolysis data of Fig. 1; the corrected V_c is 1.78 and is independent of temperature.

sealing volume, as measured by the "trapped hemoglobin" method, was grossly different at different temperatures.

The quantitative difference in results using the microhematocrit method and the "trapped hemoglobin" method is not due to different buffers, *etc.*, because the results of HOFFMAN *et al.*[19] were reproducible using the solutions and buffers of the present experiments. The discrepancy between these two sets of results is discussed later in the light of previous findings[20].

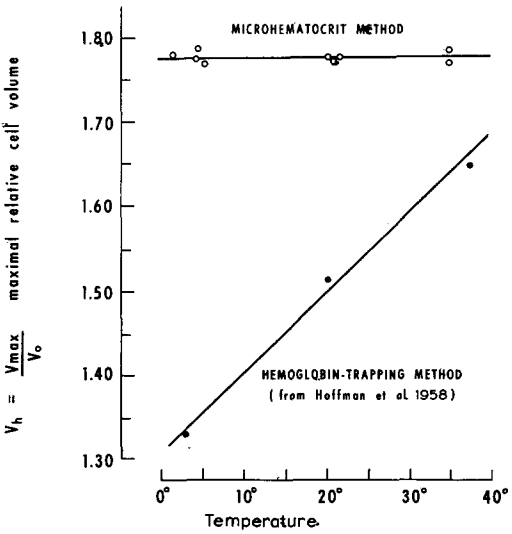

Fig. 3. A comparison between the V_c and the V_s obtained by the present microhematocrit method and by the hemoglobin-trapping method of HOFFMAN *et al.* The text explains that the microhematocrit method reflects V_c, which is independent of temperature, while the hemoglobin-trapping method monitors the sealing volume, V_s. The V_s would be lower at lower temperature because more hemoglobin would escape before membrane sealing occurred.

Events during the phase of membrane sealing

It has been found by WEED AND BOWDLER[21] that after osmotic hemolysis the ghosts become smaller than the critical hemolytic volume and subsequently reswell.

Using the Coulter counter and mean cell volume computer, which gives an mean cell volume reading for 20000 cells in 7 sec, it was possible to examine the time course of the volume changes that the erythrocyte undergoes during osmotic hemolysis. The time point chosen for each 7-sec determination was the middle of the run (3.5 sec). Typical results are shown in Fig. 4. In some experiments there was an obvious transient drop of about 5 μ^3 occurring at 25 sec, while in other experiments there was merely a plateau at 120 μ^3 before subsequent reswelling of the cell occurred. The total cell count usually did not vary by more than 1 % (see top of Fig. 4).

Effect of membrane-straining rate on hemoglobin release

An example of the effect of two different hemolysis rates on the amount of hemoglobin released is shown in Fig. 5B. Reducing the pump infusion rate from 1.36 ml/min (of 0.225 % NaCl, or 38.5 mM NaCl, in 10 mM sodium phosphate buffer, pH 7) down to 0.34 ml/min reduced the amount of hemolysis at the end of the run from 79 to 58 %. Fig. 5A shows the actual relative tonicity, T, at any moment during

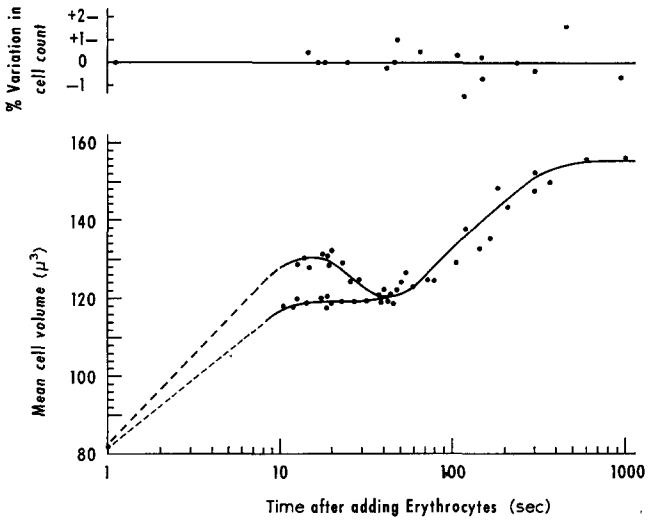

Fig. 4. Relationship between mean cell volume and time showing transient collapse and swelling of the erythrocyte ghost immediately after hypotonic hemolysis. Intact erythrocytes, with a volume of 82.5 μ^3 in 0.9% NaCl were mixed with hypotonic solution at zero time. Mean cell volume readings were taken with the mean cell volume computer. In some experiments there was a transient 5–10 μ^3 drop of the mean cell volume at 30 sec; in other experiments there was only a plateau of 120 μ^3 in this time region. The points at the top of the figure show that there was less than 1% variation in the total cell count (80000 cells/ml).

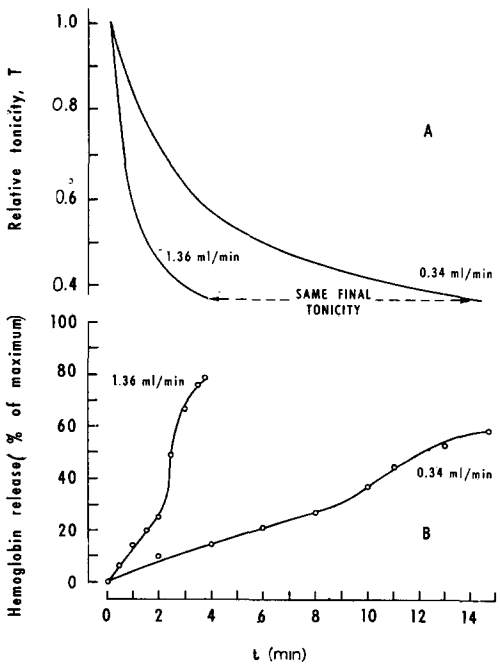

Fig. 5. Tonicity and hemoglobin release at two hemolysis rates, showing that a reduction in the rate of hemolysis reduces the amount of hemolysis even though the final tonicity is identical. (A) The actual relative tonicity, $T(=c_t/0.9\%$ NaCl, where c_t is calculated from Eqn. 1) at time t. (B) The amount of hemoglobin released from the hemolyzed cells, expressed in % of the total amount of hemoglobin in the vial. The value of 0.34 ml/min refers to the pump infusion rate in slow hemolysis.

the run; T has been calculated according to the formula described in MATERIALS AND METHODS. After the 5 ml of 38.5 mM NaCl were infused into the vial containing 1 ml of 154 mM NaCl, the final relative tonicity, T, was 0.375 % NaCl (64.1 mM NaCl) and this was the same for both infusion rates of 1.36 ml/min and 0.34 ml/min.

The effect of membrane-straining rate on V_c

The effect of two different hemolyzing rates on V_c is shown in Fig. 6. In Fig. 6A the Van Allen hematocrit reading has been converted to relative cell volume, V/V_0.

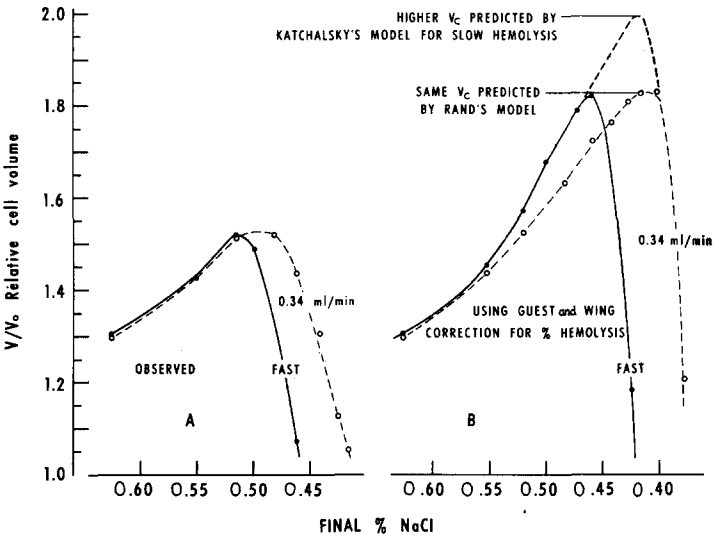

Fig. 6. Relationship of critical hemolytic volume, V_c, and membrane-stretching rate, showing that V_c is independent of the membrane-stretching rate. (A) The relative cell volumes actually observed in fast and slow hemolysis, using Van Allen hematocrit tubes. (B) The data of A corrected for % hemolysis by the GUEST AND WING method. Slow hemolysis is indicated by the pump speed of 0.34 ml/min. The same V_c of 1.825 was found for both fast and slow hemolysis, agreeing with RAND'S model that the membrane bursts at the same strain. KATCHALSKY'S model predicts a higher V_c for slow hemolysis.

Fast hemolysis here means that the 5 ml of hypotonic solution were added and mixed in a period of 2–3 sec. The fast addition of these 5 ml, therefore, represented an infusion rate of the order of 100 ml/min. The slow injection of 0.34 ml/min took 14.7 min to be completed. For the fast and slow results to be compared under similar conditions, it was also necessary to leave the "fast vial" on the magnetic stirrer for 14.7 min.

In Fig. 6B the relative volumes have been corrected for percent hemolysis using the GUEST AND WING correction method[13]. It is explicitly shown here that the V_c is between 1.82 and 1.83 and has the same value for fast and slow hemolysis. While the V_c was unaffected by the membrane-straining rate, the osmotic fragility was altered. In Fig. 6B it can be seen that reducing the infusion rate from 100 ml/min down to 0.34 ml/min reduced the osmotic fragility by approx. 0.05 % NaCl (equivalent to of the order of 0.5 atm).

The effect of membrane-straining rate on prelytic and lytic release of K^+

The effects of rapid and gradual hemolyzing rates on the prelytic leakage and lytic release of K^+ are shown in Figs. 7 and 8. Fig. 7 shows the marked reduction in

osmotic fragility during slow hemolysis, as monitored by the total release of K^+, lytic and prelytic.

In order to separate the prelytic leakage of K^+ from the lytic release of K^+, a most useful plot is one of the kind presented by KWANT AND VAN STEVENINCK[14]. This is shown in Fig. 8 where the amount of hemoglobin released is plotted *versus* the

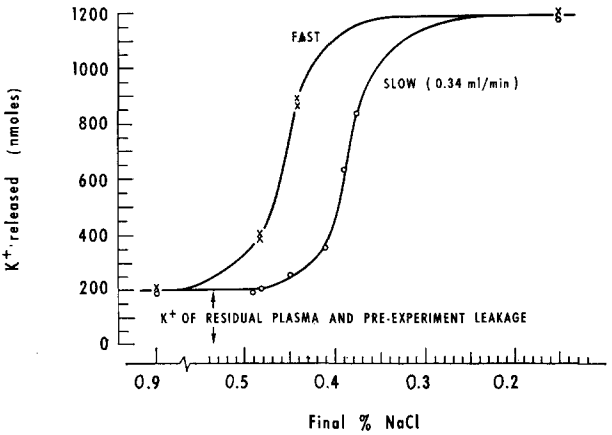

Fig. 7. Effect of fast and slow hemolyzing rates on the release of K^+ from the erythrocytes in solutions of different final NaCl concentrations. In an isotonic solution (0.9% NaCl) there were about 200 nmoles K^+, which represented the amount of K^+ from residual plasma and from K^+ leakage into isotonic solution.

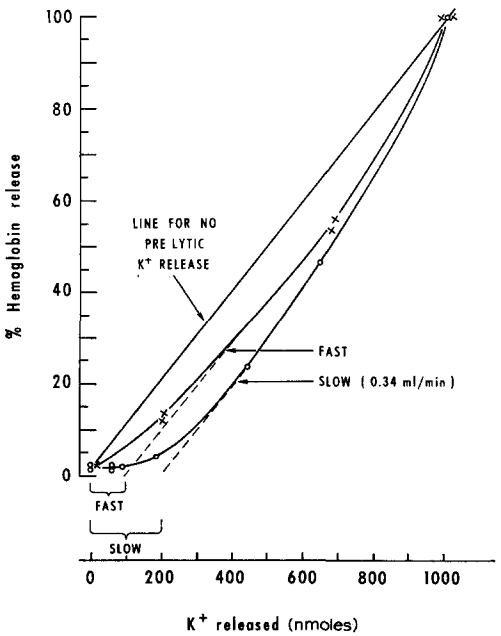

Fig. 8. Prelytic K^+ leakage in fast and slow hemolysis. The prelytic leakage of K^+ in slow hemolysis is about twice that in rapid hemolysis. The K^+ released in nmoles has been corrected for the K^+ present in the isotonic solution. If there were no prelytic release of K^+, the lytic release of hemoglobin would exactly match the lytic release of K^+. In fact, there is always relatively more K^+ released than hemoglobin; 100 nmoles in fast, and 200 nmoles in slow hemolysis.

amount of K^+ released in the same supernatant. In Fig. 8 corrections have been made for the amount of extracellular K^+ existing in the supernatant in isotonic solution (see Fig. 7).

The results in Fig. 8 indicate that for complete hemolysis there were 1000 nmoles of K^+ released altogether. In Fig. 8 the straight line drawn between 2 % (which represents the amount of benzidine-positive material in isotonic supernate) and 100 % hemoglobin release is the relation expected for a parallel release of hemoglobin and K^+ during hemolysis. As shown by many other workers[14,22–26], however, there is always a prelytic release of K^+ in hypotonic media. The K^+ released, therefore, will always be relatively more than the amount of hemoglobin released for any degree of hemolysis. This disproportionately higher release of K^+ is shown in Fig. 8 for both fast and slow hemolysis. As an index of the amount of prelytic K^+ released, a line was drawn tangential to the K^+–hemoglobin curve and extrapolated down to 2 % hemoglobin release. The tangents were taken at the point at which there was about 30 % hemolysis, since this is usually the region of greatest linearity in the standard osmotic fragility curve. The extrapolated line intercepts the abscissa at 100 nmoles for fast hemolysis and at 200 nmoles for slow hemolysis. Since the maximum K^+ release possible is 1000 nmoles, the prelytic leakages amount to 10 % (for fast hemolysis) and 20 % (for slow hemolysis) of the intracellular K^+. The prelytic leakage of 10 % in fast hemolysis agrees with the results of KWANT AND VAN STEVENINCK[14].

DISCUSSION

The results have shown (1) that the critical hemolytic volume, V_c, was constant and independent of temperature and membrane-straining rate, and (2) that the prelytic leakage of K^+ in hypotonic hemolysis increased from 10 % (of the intracellular K^+) in fast hemolysis to 20 % in slow hemolysis.

The effect of temperature on cell volumes and on osmotic fragility

The classical explanation for the reduction in osmotic fragility at elevated temperature is that given by JACOBS AND PARPART[5]. These authors proposed, based on work done by STADIE AND MARTIN[27] and VAN SLYKE *et al.*[28], that the amount of cell water (or the cell volume) was somewhat smaller at higher temperatures. The results in Fig. 1, however, show that this is not true and that erythrocytes in isotonic solution have the same volume over a wide range in temperature. The data in Fig. 1 are in agreement with those of MURPHY[10] who also found that the hematocrits of cells in isotonic solution were not significantly different at different temperatures.

It is likely that the explanation for reduced fragility at elevated temperatures is that the prelytic leakage of K^+ is increased. Normally this value of prelytic leakage is of the order of 10 % (Fig. 8 and also ref. 14). If this value were increased at higher temperatures it would result in reduced hemoglobin release or hemolysis. DAVSON[26] found that leakage of K^+ in hypotonic solution was increased at elevated temperatures. In the same way a greater prelytic release of K^+ in gradual hemolysis would seem to be the explanation for reduced fragility there (see later discussion).

Events during the phase of membrane sealing

During the period of between 10 and 40 sec after the onset of osmotic hemolysis, the mean cell volume of the hemolyzing erythrocyte remained at about 120–125 μ^3 or

reduced transiently by about 5 μ^3 before finally reswelling. These findings are compatible with those of WEED AND BOWDLER[21]. It is possible that the membrane holes, developing and sealing during the first 60 sec of osmotic hemolysis[20], may short-circuit somewhat the aperture current of the Coulter counter. Fig. 9A is a sketch of 3 individual erythrocytes hemolyzing at different critical hemolytic volumes and at different time courses. Each cell is depicted as first swelling rapidly in hypotonic solution and bursting at V_c; the ghost then either collapses slightly or else the membrane holes short-circuit the aperture current; finally the ghost reswells[21] or the membrane holes slowly seal. Fig. 9B is the time course of development and sealing of membrane holes[20]. Fig. 9C is the composite of Fig. 9A and is roughly what is seen experimentally in Fig. 4.

The effect of temperature on V_c

The microhematocrit results in Figs. 1, 2 and 3 indicate that the critical hemolytic volume, V_c, was the same over a wide range of temperature. Since the erythrocyte is a sphere immediately before hemoglobin release[4,15,29], this suggests that the cell membrane area was roughly constant at different temperatures.

The explanation for nonidentical values between the GUEST AND WING V_c and the HOFFMAN V_s (see Fig. 3) may be either of the following.

(1) The "hemoglobin-trapping" method measures the sealing volume (V_s) of the

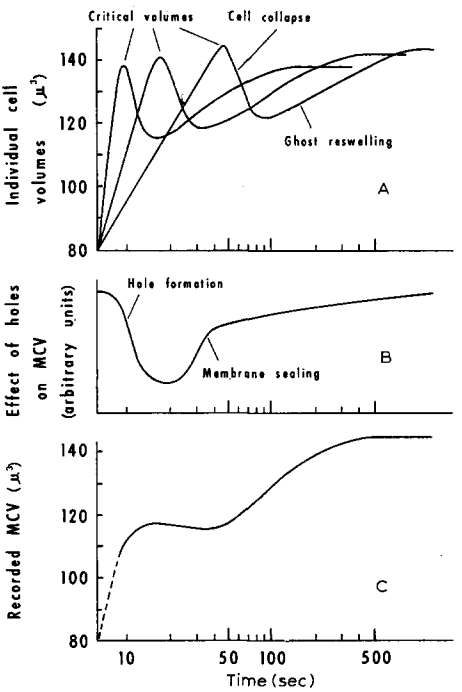

Fig. 9. A possible explanation of the time course of the volume changes observed in the results of Fig. 4. (A) Three individual erythrocytes as swelling to, and bursting at, different critical hemolytic volumes; the cells then either collapse slightly or the membrane holes (B) short-circuit the aperture current before the cell finally reswells. (B) The time course of the development and sealing of membrane holes (taken from SEEMAN[20]). (C) The postulated composite for a population of cells (compare with Fig. 4). MCV, mean cell volume.

ghost and this value may be affected by the amount of cell collapse that occurs before the ghost membrane seals itself. As the temperature is lowered, the sealing rate is greatly reduced, as shown by the work of HOFFMAN *et al.*[30]; the cell will have more time to collapse down before sealing, thus leading to a lower V_s at lower temperature; the V_c would be unaffected.

(2) The second possibility is that during hemolysis the hemoglobin does not come to complete diffusion equilibrium at the different temperatures. This would only affect V_s and not V_c.

The effect of temperature on the egress of hemoglobin has not yet been studied[31]. It is conceivable that under some experimental conditions the sealing may be very rapid such that the cell membrane seals before there has been time for the hemoglobin to exit. This may be the explanation for the observation that dextran does not alter the osmotic fragility but does reduce the amount of hemoglobin escaping[32].

Effect of membrane-straining rate on V_c

The results shown in Fig. 6 indicate that the V_c has the same value in slow as in fast hemolysis. This finding is in agreement with the work of RAND[3,4], who proposed that the erythrocyte hemolyzes at the same membrane strain, regardless of the tension inside the cell or in the membrane. The values for the GUEST AND WING-corrected V_c in Fig. 6 is about 1.82 for both fast and slow hemolysis. As shown at the top of Fig. 6, the V_c that would be expected according to the viscoelastic model of KATCHALSKY *et al.*[1] would be around 2.0.

While the V_c appears to be unaffected by the hemolyzing rate, there is no question that the membrane-straining rate is drastically reduced in slow hemolysis. This can be shown as follows. Eqns. 4 and 5 in the paper of KATCHALSKY *et al*[1]. give membrane-strain rate, de/dt, as

$$\frac{de}{dt} = \frac{1}{r_s} \cdot \frac{dV}{A \cdot dt} \tag{2}$$

where e is the membrane strain ($= (r - r_s)/r_s$), where r is the radius of the hemolyzing and spherical erythrocyte, where r_s is the radius of the quasispherical erythrocyte on appearance of stresses in the membrane, where A is the area of the erythrocyte and where dV/dt is the rate of cell swelling.

The relative rate of cell swelling may be obtained from the osmometer formula of PONDER[15],

$$V = V_0 W_{eff} \left(\frac{1}{T} - 1\right) + 1 \tag{3}$$

where V is the volume of the cell, V_0 is the volume of the cell in isotonic solution (154 mM NaCl), where W_{eff} is the effective fractional water volume of the cell and where T is the relative tonicity and is equal to the NaCl concentration divided by 154 mM NaCl. The rate of cell swelling will be:

$$\frac{dV}{dt} = \frac{-V_0 \cdot W_{eff}}{T^2} \cdot \frac{dT}{dt} \tag{4}$$

The membrane-strain rate will thus be:

$$\frac{de}{dt} = \frac{-V_0 \cdot W_{eff}}{r_s \cdot A} \cdot \frac{1}{T^2} \cdot \frac{dT}{dt} \tag{5}$$

In a first-order approximation, the factor, $(V_0 \cdot W_{eff}) / (r_s \cdot A)$, may be considered to be virtually constant during the course of swelling. The membrane-straining rate, therefore, will be proportional to

$$\frac{-1}{T^2} \cdot \frac{dT}{dt} \tag{6}$$

This Factor 6 is plotted in Fig. 10. The values for T have been taken from the data in Fig. 5A. It can be seen from the results of Fig. 10 that the drop in membrane-straining rate from fast hemolysis down to 0.34 ml/min is of the order of 1000-fold.

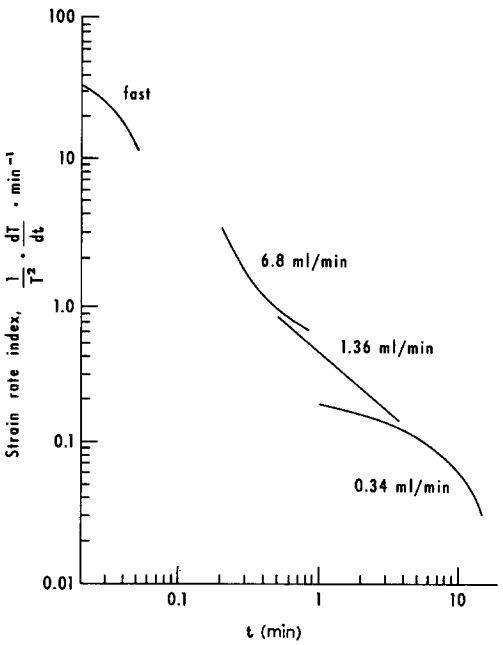

Fig. 10. Showing how the strain rate index, $(-1/T^2) \cdot (dT/dt)$, depends on both the pump infusion rate and the duration of the infusion period. The index has been derived from equations of KATCHALSKY *et al.*[1] and PONDER[15]. The index is positive since dT/dt is negative. All values have been worked out using 0.225% NaCl as the solution being infused.

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REFERENCES

1 A. KATCHALSKY, O. KEDEM, C. KLIBANSKY AND A. DE VRIES, in A.L. COPLEY AND G. STAINSBY, *Flow Properties of Blood and other Biological Systems*, Pergamon Press, New York, 1960, p. 155.

2 D. DANON, A. NEVO AND Y. MARIKOVSKY, *Bull. Res. Council Israel, Sect. E*, 6 (1956) 36.
3 R. P. RAND, *Biophys. J.*, 4 (1964) 303.
4 R. P. RAND, *Federation Proc.*, 26 (1967) 1780.
5 M. H. JACOBS AND A. K. PARPART, *Biol. Bull.*, 60 (1931) 95.
6 M. H. JACOBS, H. N. GLASSMAN AND A. K. PARPART, *J. Cellular Comp. Physiol.*, 7 (1936) 197.
7 A. K. PARPART, P. B. LORENZ, E. R. PARPART, J. R. GREGG AND A. M. CHASE, *J. Clin. Invest.*, 26 (1947) 636.
8 E. MORTENSEN, *Acta Med. Scand.*, 173 (1963) 683.
9 E. MORTENSEN, *Acta Med. Scand.*, 173 (1963) 693.
10 J. R. MURPHY, *J. Lab. Clin. Med.*, 69 (1967) 758.
11 H. KOEPPE, in E. PONDER, *Hemolysis and Related Phenomena*, Grune and Stratton, New York, 1948, p. 56.
12 L. J. BEILIN, G. J. KNIGHT, A. D. MUNRO-FAURE AND J. ANDERSON, *J. Gen. Physiol.*, 50 (1966) 61.
13 G. M. GUEST AND M. WING, *J. Clin. Invest.*, 21 (1942) 257.
14 W. O. KWANT AND J. VAN STEVENINCK, *Biochem. Pharmacol.*, 17 (1968) 2215.
15 E. PONDER, *Hemolysis and Related Phenomena*, Grune and Stratton, New York, 1948, p. 84.
16 F. C. BING AND R. W. BAKER, *J. Biol. Chem.*, 92 (1931) 589.
17 J. V. DACIE, *Practical Hematology*, Churchill, London, 2nd Ed., 1956, p. 140.
18 P. G. HATTERSLEY AND D. RAGUSA, *Am. J. Clin. Pathol.*, 37 (1967) 229.
19 J. F. HOFFMAN, M. EDEN, J. S. BARR, Jr. AND R. H. S. BEDELL, *J. Cellular Comp. Physiol.*, 51 (1958) 405.
20 P. SEEMAN, *J. Cell Biol.*, 32 (1967) 55.
21 R. I. WEED AND A. J. BOWDLER, *J. Clin. Invest.*, 45 (1966) 1137.
22 S. E. KERR, *J. Biol. Chem.*, 85 (1929) 47.
23 J. E. HARRIS, *J. Biol. Chem.*, 141 (1941) 579.
24 E. J. ROBINSON AND E. PONDER, *Biochem. J.*, 28 (1934) 1940.
25 E. PONDER, *Cold Spring Harbor Symp. Quant. Biol.*, 8 (1940) 133.
26 H. DAVSON, *J. Cellular Comp. Physiol.*, 10 (1937) 247.
27 W. C. STADIE AND K. A. MARTIN, *J. Biol. Chem.*, 60 (1924) 191.
28 D. D. VAN SLYKE, H. WU AND F. C. MCLEAN, *J. Biol. Chem.*, 56 (1923) 765.
29 R. P. RAND AND A. C. BURTON, *J. Cellular Comp. Physiol.*, 61 (1963) 245.
30 J. F. HOFFMAN, D. C. TOSTESON AND R. WHITTAM, *Nature*, 185 (1960) 186.
31 J. F. HOFFMAN, *J. Gen. Physiol.*, 42 (1958) 9.
32 H. G. DAVIES, N. V. B. MARSDEN, S. G. OSTLING AND A. M. M. ZADE-OPPEN, *Acta Physiol. Scand.*, (1968) in the press. Quoted in A. M. M. ZADE-OPPEN, *Some Aspects of Osmotic Hemolysis*, Acta Universitatis Upsaliensis, Uppsala, 1968.