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Effect of Cell Geometry, Internal Viscosity, and pH on Erythrocyte Filterability (41089)[1]

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Abstract. A simple filtration method has been developed for measuring the passage time of dilute erythrocyte suspensions through 2.8-μm cylindrical pores relative to the passage time of the suspending buffer. This method of determining relative filtration time (RFT) is shown to be sensitive to the major determinants of overall red cell deformability by demonstration of the effects of variations in internal viscosity (intracellular hemoglobin concentration), cell geometry (volume, surface area-to-volume ratio), and membrane properties. These factors were examined by varying the buffer pH at constant osmolality and by varying the buffer osmolality at constant pH. In addition, the influence of cell volume alone was investigated by measuring RFT of suspensions of rat, rabbit, and human erythrocytes, of naturally different volumes but of the same basic biconcave shape. The results of these studies indicate that: (a) RFT is significantly increased by decreases in the surface area-to-volume ratio produced by osmotically induced cell swelling; (b) RFT increases nearly linearly with increasing intracellular hemoglobin concentration of cells in hypertonic buffers; (c) reduction of buffer pH below 7.20 causes a significant increase in RFT that is not explained by cell volume or geometric changes and is presumably due to altered membrane properties; and (d) the contribution of cell volume alone in normal biconcave erythrocytes over the range of 60–100 μm^3 is much less than that of even slight decreases in the surface area-to-volume ratio. These studies illustrate well the relative contributions of each of the major determinants of red cell deformability and the amount of information that can be obtained using a relatively simple filtration method.

The ability of erythrocytes to deform is necessary for the performance of their function in oxygen delivery. Both the release of young erythrocytes from the bone marrow and the removal of senescent cells by the spleen depend on this ability of red cells to traverse narrow slits or pores. Severe alterations in deformability may prevent the maintenance of adequate circulating levels of viable, functioning erythrocytes and thereby produce clinical disorder. Minor alterations may provide clues to subtle metabolic or membrane abnormalities associated with inherited or acquired systemic disease.

Overall erythrocyte deformability is influenced by cell geometry (in particular the surface area-to-volume ratio), internal viscosity (largely a function of the concentration and physiochemical state of hemoglobin), and the properties of the erythrocyte membrane (1). A method for measuring red cell deformability under a variety of *in vitro* conditions or in various disease states should be able to distinguish to some extent the contributions made by each of these factors, both to clarify the nature of any differences observed among cell populations and to provide information helpful in determining the most fruitful direction to take in further experimentation.

Descriptions of methods used to measure deformability of red cells often do not include a demonstration of how the experimental results might be influenced by alterations in geometry, internal viscosity, or membrane properties. Such an understanding and elucidation of the limitations and advantages of any method would seem essential for the proper interpretation of significant findings.

We report here an analysis of the effects of several factors on the passage time of

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dilute erythrocyte suspensions through polycarbonate membrane filters with 3-μm cylindrical pores. Even a relatively simple filtration method such as that described in this article and by others (2–4) can be used to assess the relative contributions of cell size, geometry, and viscosity to overall deformability.

Materials and Methods. *Preparation of blood.* Blood samples were obtained from the antecubital veins of healthy human volunteers,[2] the marginal ear veins of rabbits, and the abdominal venae cavae of anesthetized rats. Heparin (10 U/ml) was the anticoagulant in all cases.

Suspending media. Phosphate buffers (pH 7.40) of varying osmolalities were prepared by dilution from a standard stock solution of the following composition in g/liter: NaCl, 90.0; Na_2HPO_4, 13.66; NaH_2PO_4; H_2O, 2.43. Buffer osmolalities in the experiments described here ranged from 200 to 600 mosm/kg, measured using either freezing-point depression (Fiske Osmometer, Fiske Associates, Inc., Hawthorne, Mass.) or vapor pressure (Wescor Model 5100B Vapor Pressure Osmometer, Wescor, Inc., Logan, Utah).

Isotonic (290 mosm/kg) phosphate buffers of varying pH's were prepared by mixing of two stock solutions of the following compositions in g/liter: solution A: $NaH_2PO_4 \cdot H_2O$, 1.4112, NaCl, 8.55; solution B: Na_2HPO_4, 1.454, NaCl, 8.40. Buffer pH's in the experiments described below ranged from 6.5 to 8.0.

Filters. Polycarbonate sieve filters, 13 mm in diameter with a nominal pore size of 3 μm, were obtained from Nuclepore Corporation (San Francisco, Calif.). All filters used in these experiments were from a single batch (No. 04448) with an actual pore size of 2.75–2.85 μm and a pore density of $1.9 \cdot 10^6/cm^2$. The filters were soaked in isopropanol for 30 min and rinsed with distilled water prior to use in an experiment.

Determination of the relative filtration time (RFT). The filtration apparatus used is shown diagrammatically in Fig. 1. The entire apparatus, reservoirs of suspending media, cell suspensions, pipettes, etc., were contained within an environmental chamber at a temperature in the range of 35–37°. All measurements of filtration were done in this chamber.

The following protocol was used to obtain a value of RFT for a given cell suspension. A filter soaked and washed as described above was mounted in a 13-mm polycarbonate "Pop-Top" filter holder (Nuclepore) with the shiny side of the filter toward the lower, outflow end of the holder, and the holder was filled with buffer to overflowing from below through a three-way stopcock. A modified 1.0 ml-Van Slyke–Neill pipet (Arthur H. Thomas Co.,

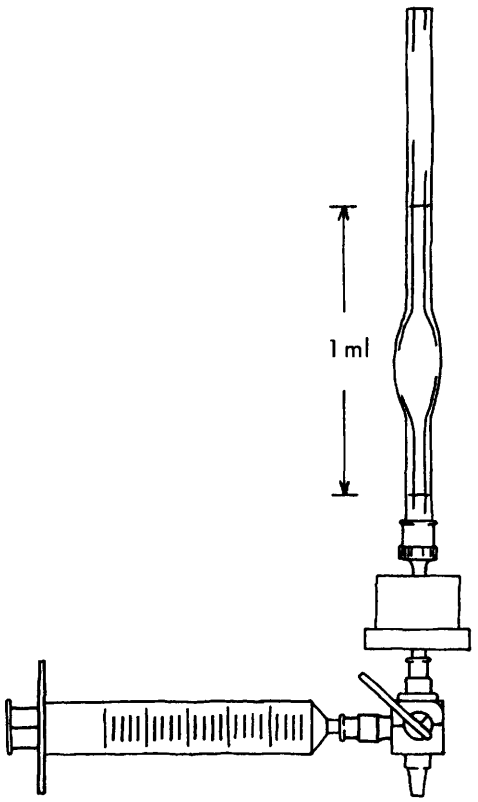

FIG. 1. Diagram of filtration apparatus used in the experiments described in this paper, consisting of a modified Van Slyke pipet, polycarbonate sieve filter holder, three-way stopcock, and flushing syringe. For description of experimental procedure, see text.

[2] Informed consent was obtained from all human subjects and all procedures were performed according to the Declaration of Helsinki and with the approval of the Committee for the Protection of Human Subjects of the Maine Medical Center.

Philadelphia, Pa.), fitted with a male luer connector, was filled with buffer and attached to the top of the filter holder with the outflow stopcock in the closed position. When the stopcock was opened, the buffer flowed by gravity through the filter and the time required for the passage of 1.0 ml was measured with a stopwatch and recorded. This procedure was repeated until three consecutive measurements of the "buffer time" were obtained that agreed to within 0.1 sec. When a satisfactorily constant "buffer time" was obtained, the pipet was filled with the dilute cell suspension prepared in that buffer and the time for passage of 1.0 ml of this suspension was measured and recorded. The filter in its holder was subsequently rinsed free of cells by flushing with buffer, washed in distilled water in an ultrasonic bath for 1 min, and rinsed again with buffer. This entire protocol was repeated with three different filters to obtain triplicate measurements on each cell suspension.

The RFT was calculated by dividing the filtration time for the cell suspension by the buffer time for that filter and ultimately averaging the resulting values for RFT obtained with the three filters. By thus com paring the filtration times of the cell suspension and buffer for each filter, the measurement variability among the filters used in an experiment was greatly reduced.

Although values for RFT could conceivably range from 1.0 (no increase in filtration time due to the presence of cells in the buffer) to infinity (cell suspension will not pass through the filter), only RFTs of less than 10 were considered for analysis to avoid any time-dependent artifacts such as sedimentation or aggregation of erythrocytes.

Preparation of cell suspensions. Cell suspensions were prepared in phosphate buffers of varying osmolality by direct dilution of whole blood into buffer. The volume of the original blood sample necessary to produce a 0.5% hematocrit in 20 ml of isotonic (290 mosm/kg) buffer was calculated; this volume was then used to prepare all the dilutions at other osmolalities to assure equal cell concentrations (number of cells per unit volume) in all the suspensions. These dilute cell suspensions were prepared 5 min before the filtrations were to be performed in order to allow osmotic equilibration to occur.

Cell suspensions were prepared in buffers of varying pH's in a similar manner to assure equal cell concentrations in all suspensions.

Mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) were calculated for the initial whole blood sample and for each of the cell suspensions used in an experiment. Following measurement of RFT, the remainder of the dilute cell suspension was concentrated to hct 20–30% by low-speed centrifugation, removal of a large portion of the suspending buffer, and gentle resuspension of the cells. Erythrocytes were counted using a Coulter counter (Model Fn, Coulter Electronics, Hialeah, Fla.); hemoglobin concentration was determined using the cyanmethemoglobin method and commercially available standards (Hycel, Inc., Houston, Tex.); hematocrit was measured using a microhematocrit centrifuge with no correction applied for trapped plasma.

Data analysis. Statistical analysis of data was accomplished using prerecorded programs supplied for the HP-67 programmable calculator (Hewlett–Packard, Corvallis, Ore.); these were used for comparison of means, linear regression, and one-way analysis of variance. Supplementary programs for curvilinear and multiple regression were written for the calculator using formulae from statistical reference texts (5–7).

Results. *Determination of RFT.* The calculation of a mean RFT for a cell suspension from triplicate determinations using three filters serves to reduce the variability arising from slight differences in pore size or density among the filters in a given batch. This point is addressed in Table I, summarizing the experimental data from 15 experiments in which RFT was determined on cell suspensions prepared from blood of healthy nonsmoking human volunteers. The intrinsic differences among the 45 different filters utilized in these 15 experiments are indicated by the high coefficient of variation accompanying the mean buffer

TABLE I. EXPERIMENTAL VARIABILITY IN RFT MEASUREMENT

	Buffer time[a]	Cell time[b]	RFT[c]
n	45	15	15
Mean	4.95	11.33	2.30
SD	±0.66	±1.48	±0. 19
c.v. (%)	13.3	13.1	8.3

[a] Each of the three filters in each of the 15 experiments (45 different filters) can be considered independently in determining the variability among buffer times.

[b] Time for passage of 1 ml of cell suspension, determined for each of the 15 experiments as the average of triplicate determinations within that experiment using three different filters.

[c] RFT determined for each of the 15 samples from triplicate determinations on a single cell suspension using three filters, as described in the text.

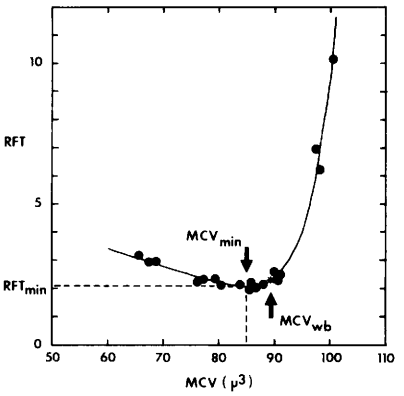

FIG. 2. Relationship of RFT to osmotically induced changes in MCV. All data points were obtained on cell suspensions prepared from the blood of a single healthy human volunteer. The solid line was generated using a curve-fitting routine described in the Appendix. The vertical and horizontal dashed lines indicate the location of RFT_{min} and MCV_{min} as indicated. The asterisk and arrow indicate the location of the MCV_{wb} for comparison.

time of 4.95 sec. The similar degree of variability around the mean value for the "cell time" determined for the 15 subjects reflects these differences as well. The coefficient of variation for the RFT determination is much less, indicating the benefit of calculating the ratio of cell time to buffer time for *each* filter. Any such reduction in the experimental variability within a group enhances the ability of the method to detect differences among groups of subjects due to the properties of the cells in the suspensions.

Effect on RFT of osmotically induced changes in erythrocyte geometry and internal viscosity. The most meaningful method of expressing the data obtained from these experiments is shown in Fig. 2. Varying the osmolality of the suspending buffers produces erythrocyte volume changes that can be related to the RFTs of these suspensions by a curve fit to the data using an empirical formula (see Appendix). Analysis of this curve reveals a minimum RFT (RFT_{min}) and a corresponding minimum MCV (MCV_{min}). These two parameters mark the point of optimization of the filterability of the particular cell suspension with respect to changes in cell volume. It should be noted in Fig. 2 that relatively small increases in MCV (5–10% above the MCV measured in whole blood [MCV_{wb}]) produce large increases in RFT and that *decreases* in MCV also produce increases in RFT, although not as dramatic.

The fact that these osmotically induced changes in RFT reflect more than simply erythrocyte size is shown in Fig. 3. Rat, rabbit, and human red cell populations with MCV_{wb}'s of approximately 65, 72, and 90 μm^3, respectively, demonstrate MCV/RFT curves that are very similar in shape but separated on the MCV axis (top panel). However, a clearly different relationship exists between RFT and MCV_{wb} for normal biconcave cells from all three species (lower panel). It appears from these data that the normal geometry of mammalian erythrocytes provides maximum deformability and that deviations in either direction (swelling or shrinking) diminish the ability of these cells to pass through cylindrial channels. Since the MCV_{wb}'s of the three species are quite different, the geometric factor responsible for the sharp increase in RFT with cell swelling must be the surface area-to-volume ratio (SA/V), as suggested previously (8, 9).

The normal optimization of erythrocyte deformability is further illustrated in Fig. 4, which depicts the relationship between MCV_{wb} and MCV_{min} for cell suspensions

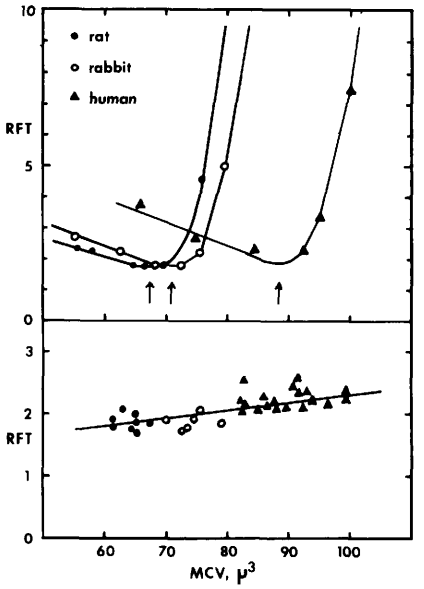

FIG. 3. Upper panel: Relative filtration times for suspensions of osmotically manipulated erythrocytes from rats, rabbits, and humans. Each of the curves was generated from a single blood sample. The vertical arrows indicate MCV_{min} for each of the species. Lower panel: Relationship between MCV and RFT for normal biconcave erythrocytes from rats, rabbits, and humans. The solid line was fitted to the points using the least-squares regression technique and represents the equation: RFT = 1.06 + (0.0124) (MCV) where r = 0.4635 and n = 33 ($P < 0.01$). The rat and rabbit erythrocytes were suspended in buffer at 310 mosm/kg, a level isotonic to the plasma of these animals as measured in our laboratory.

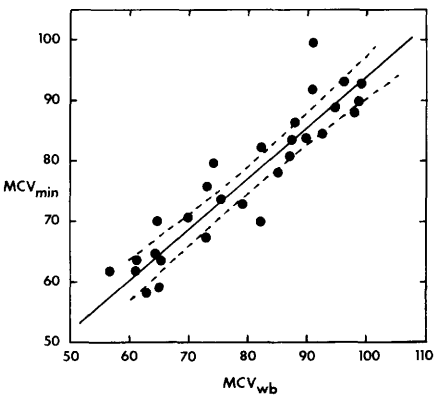

FIG. 4. Relationship of MCV_{wb} to MCV_{min}. Each symbol indicates the values determined on a single blood sample from a rat, rabbit, or human as indicated in Figs. 2 and 3. The solid line was fit to the data using the least-squares linear regression method and the dashed lines indicate the 95% confidence limits.

from the three species. The regression line has a slope quite close to (and not significantly different from) unity, indicating that the two variables are nearly identical.

RFT_{min}, like RFT, is not completely independent of cell size. Figure 5 illustrates the relationship between MCV_{min} and RFT_{min} for suspensions from rats, rabbits, and humans. A significant relationship does exist between the two variables with RFT_{min} increasing in a nonlinear fashion as MCV_{min} is increased. This is similar to the relationship between MCV_{wb} and RFT (measured in isotonic buffer) shown in Fig. 3, lower panel.

Explanation of the results shown in Figs. 4 and 5 involves a discussion of the primary determinants of erythrocyte deformability: membrane properties, SA/V, and internal viscosity (1, 10). Osmotically induced changes in erythrocyte volume affect internal viscosity (by concentration or dilution of hemoglobin) and SA/V. No evidence exists that intrinsic membrane properties are altered by this treatment and recent evidence from micropipet (11) and rheological studies (12–14) indicates that membrane physical and mechanical properties may play only a minor role in determining overall cell deformability.

Swelling of erythrocytes in hypotonic

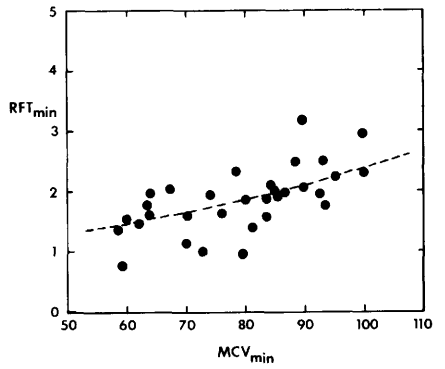

FIG. 5. Relationship of MCV_{min} to RFT_{min}. Each symbol represents the values determined on a single blood sample for one of the three species. The dashed curve represents the power curve $(RFT_{min} - 1) = 8.3 \cdot 10^{-5}(MCV_{min})^{2.111}$ where r^2 = 0.5668 and n = 32.

media decreases SA/V (tending to reduce cell deformability) while at the same time decreasing MCHC and, therefore, internal viscosity (tending to increase cell deformability). Osmotic shrinking of erythrocytes would have the opposite effect in both cases. At some point, an optimum combination of SA/V and internal viscosity produce a maximally deformable cell; according to the results presented here, this optimum point lies close to or at the normal state of the erythrocyte.

As mentioned above, the decrease in SA/V that occurs with cell swelling appears to be responsible for the increase in RFT. Evidence that the less-striking increase in RFT with cell shrinking is due to increasing MCHC is shown in Fig. 6. A highly significant ($P < 0.01$) nonlinear relationship exists between RFT and MCHC in suspensions of cells from all three species in buffers with osmolalities greater than 300 mosm/kg.

Effect of pH on RFT. The results of five experiments in which RFTs were measured on human erythrocytes suspended in isotonic buffers ranging in pH from 6.5 to 8.0 are shown in Fig. 7. The overall effect of pH on RFT is significant ($P < 0.05$, one-way analysis of variance) and mean RFTs at pH's of 6.5, 6.8, and 7.0 are significantly different from the control value at pH 7.40 ($P < 0.05$, t-test for paired data). Since RFT is sensitive to erythrocyte volume changes as demonstrated above, the effect of pH on red cell indices in this group of experiments was examined as well. The results, summarized in Table II, indicate a significant and reciprocal effect of pH on MCV and

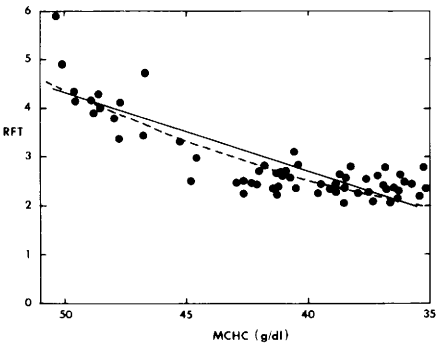

FIG. 6. Relationship of RFT and MCHC. Each symbol represents values for RFT and MCHC obtained from cell suspensions in buffers with osmolalities greater than 300 mosm/kg, prepared from blood samples from all three species. The solid line is analogous to the linear slowly rising left portion of the curves in Fig. 3; the dashed line fit to the data points represents the power curve $(RFT - 1) = 2.46 \cdot 10^{-6}(MCHC)^{3.611}$ where $r^2 = 0.7609$ and $n = 62$.

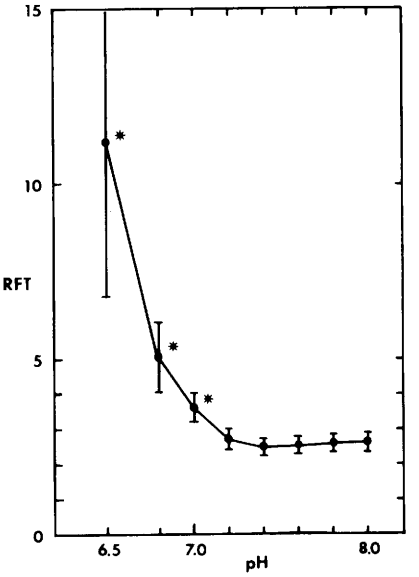

FIG. 7. Influence of pH on RFT. Symbols indicate mean RFT ± SD for five experiments. The overall effect of pH on RFT was statistically significant ($F(7,27) = 3.367$ for one-way analysis of variance); asterisks indicate RFTs that are significantly different from the control RFT at pH 7.40 ($P < 0.05$ using the t test for paired samples).

TABLE II. EFFECT OF pH ON MCV AND MCHC[a]

pH	MCV[b] (μ^3)	MCH (pg)	MCHC[b] (g/dl)
6.5	101.3 ± 4.1	31.67 ± 1.41	31.28 ± 1.07
6.8	96.7 ± 5.8	31.61 ± 1.32	32.08 ± 1.44
7.0	97.3 ± 6.4	31.47 ± 1.06	32.40 ± 1.17
7.2	94.6 ± 7.2	31.64 ± 1.56	33.50 ± 0.84
7.4	92.2 ± 3.0	31.78 ± 1.33	34.49 ± 1.17
7.6	92.5 ± 6.4	31.64 ± 1.89	34.53 ± 1.09
7.8	90.5 ± 6.1	31.73 ± 1.31	35.14 ± 1.32
8.0	88.3 ± 5.9	31.52 ± 1.00	35.78 ± 1.29

[a] Values are mean ± SD for five experiments.
[b] Significant effect of pH ($P < 0.05$, one-way analysis of variance).

MCHC, with MCH unaffected as expected. To examine whether the observed increases in MCV at the low pH's could account for the increased RFT values in these samples, a group of experiments were performed in which the same blood sample was used to prepare suspensions of cells both in pH 7.40 buffers at varying osmolalities and in 290 mosm/kg buffers of varying pH's. The results of one of these experiments are shown in Fig. 8; clearly different relationships between MCV and RFT are obtained in the two cases, with the increases in RFT produced by low pH greater than could be expected on the basis of a simple increase in cell size.

Discussion. The experimental method and the results of *in vitro* studies described here reveal the relative contributions of erythrocyte size, geometry, and internal viscosity to the measurements of cell deformability using filtration techniques. Cell size (as indicated by MCV) does not greatly affect RFT even over the wide range of 60–100 μm^3, as long as the erythrocytes possess the normal biconcave shape. Conceptually similar results were obtained in a study of blood viscosity in various species (15). Measurements of erythrocyte deformability in 25 mammalian species using the ektacytometer support this conclusion as well (16). Regardless of size, all normally biconcave mammalian red cells were found to deform under shear stress to a minimum diameter of less than 3 μm. In the present study, alterations in the shape, especially decreases in SA/V, have a pronounced effect on RFT that strongly suggest a significant reduction in overall deformability; this effect is equally important regardless of the normal volume of the biconcave cell (see Fig. 3). Although the geometric alterations in this study were produced osmotically, the possibility exists that similar results would be obtained if the abnormality were genetic, the result of systemic pathology, or drug induced. For example, a large difference between RFTs measured on two isotonic cell suspensions whose MCVs and MCHCs are similar means that geometric or membrane-related differences must be present. Conversely, the *lack* of a large difference in RFT between two cell suspensions with differing MCVs but similar MCHCs would not be surprising, and would indicate that the larger cells may be as deformable as the smaller due to the preservation of a favorable SA/V ratio. Thus, knowledge of erythrocyte indices and the measurement of RFT as described here in an isotonic buffer can indeed shed light on the possible reasons for altered erythrocyte deformability.

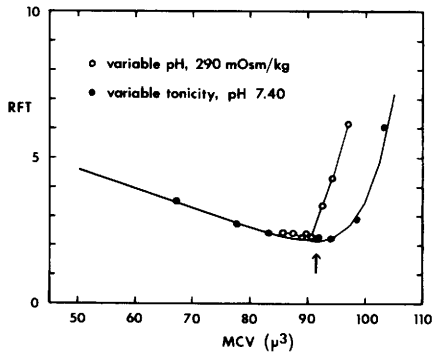

FIG. 8. Comparison of the effect on RFT of MCV changes produced by varying either the osmolality or the pH of the suspending buffer. Closed circles indicate osmotically induced changes in MCV; open circles indicate pH-induced MCV changes. The vertical arrow indicates the MCV_{min}, calculated as described in the Appendix.

The significant effect of pH on RFT is not completely explained by altered cell geometry or internal viscosity (Fig. 8). The physical properties of the erythrocyte membrane proteins may be sufficiently affected by the reduction in pH to account for the observed pattern, as has been suggested previously (17). This pH-induced change in deformability may very well be related to sequestration of erythrocytes in the acidic environment of the splenic sinusoids or to the abnormal erythrocyte flow patterns observed in human diabetic ketoacidosis (18).

The value of an analysis of an experimental method such as described here lies not only in its contribution to the understanding of results obtained from filtration studies of pathologically altered erythrocytes but also in its generation of data suggesting additional avenues of research into the pathogenesis or properties of abnormal red cells.

Appendix. The curves fit to the data in Figs. 2, 3, and 8 represent the following empirical equation:

$$(RFT - 1) = a(MCV) + b(MCV)^c$$

For the curve-fitting procedure, a value for c was estimated, then a and b were calculated using a multiple-regression program written for the HP-67 programmable calculator using standard formulas (7). This process was repeated until a minimum value for r^2 was obtained for the relationship. MCV_{min} was obtained by differentiation of the above formula following insertion of the numerical values for the coefficients; RFT_{min} was determined by substitution of MCV_{min} into the original equation. Six pairs of values for RFT and MCV were found to be the best combination of accuracy in fitting the curve and ease in performing the experiment.

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