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Cell-Membrane and Rheological Mechanisms in Dynamic
Osmotic Hemolysis and Repair of Erythrocytes and Ghosts
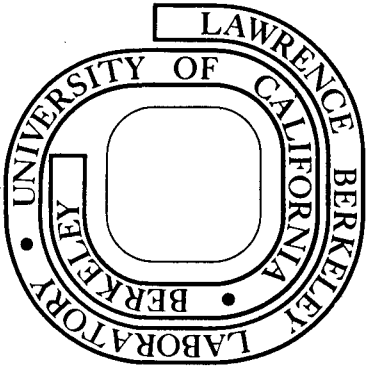
by

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Abstract. Normal human erythrocytes undergoing abrupt osmotic hemolysis display a single, transient, localized "blowout" over 15 to 20% of their total surface area. An immediate and striking drop in apparent volume for such cells, as measured by electronic volume spectroscopy (resistive pulse spectroscopy, RPS), is ascribed primarily to a greatly increased, flow-induced, cell-membrane deformation and associated expulsion of "ghost" contents rather than to an intrinsic property of hemolysis per se. The time and flow-rate dependence of the RPS spectra measure the osmotic and mechanical repair of the hemolytic lesion in the membrane. Restored ghosts rehemolyze at a critical volume and lesion size similar to those of the original lysing cells. Membrane rather than cytoplasmic (internal viscosity) properties dominate deformability measured from RPS spectral shape.

Mechanisms involved in the failure of the red blood cell membrane under osmotic stress, and in its recovery, are of interest in a variety of pure and applied biomedical areas. Although such osmotic hemolysis has long been studied (1), many aspects of the phenomenon have remained unclear. The technique of resistive pulse spectroscopy (RPS), recently developed for assaying cell-membrane deformability and for improving resolution of cell-size distributions (2), offers new opportunities to study hemolytic mechanisms and membrane properties per se (3). We report herein new results on the following: (i) the nature and size of the lesion in abrupt osmotic hemolysis; (ii) the kinetics of formation of ghosts from intact cells swollen to critical volume; (iii) the dynamics of ghost-membrane lesion recovery and repair; and (iv) the osmotic, rheological, and rehemolyzing properties of isotonically restored ghosts. In all of this work, glutaraldehyde (glut.) responses of both intact cells and ghosts play an important role.

Cells subjected to osmotic hemolysis have been variously reported to leak selectively (4,5,6), to burst suddenly (7,8), or to do both (9). The different experimental conditions used by different observers render difficult any simple generalization of mechanism. In a recent review, Seeman leans toward a local-lesion interpretation but indicated that the question is still not settled (10). Baker and Gillis (11) used glutaraldehyde treatment to trap up to 80% of cells in a state of local hemolysis. Although their microscopic pictures could be taken as evidence of a local-lesion event occurring during normal osmotic hemolysis, these investigators believed instead that these observations represent a glutaraldehyde-chemical artifact.

From our initial studies on the interactions of intact
and hemolyzing red blood cells (RBC) with glutaraldehyde, we
have been able to select simple conditions to effectively
arrest cell populations in predictable stages of swelling and
lysis, including up to 100% undergoing hemolysis. Cells caught
in the act of hemolysis show a volcano-like eruption through
a single, circular, localized site (Fig. 1). Measurements
made on 33 of these "neonate" ghosts (cells lysed in hypotonic
solutions) indicate that the lunar surface areas corresponding
to the hemolytic lesion occupy about 19% of the total surface
area, i.e., a lesion area of $27\mu^2 \pm 6$ (s.d.), based on an initial
total surface estimated at $140\mu^2$ (12).

The RPS analysis technique is based on the extension of
conventional Coulter-type counting-sizing principles (13).
Using this technique, along with additional microscopic obser-
vations and experiments clarifying the role of glutaraldehyde,
we have acquired evidence that the large local lesions seen
in Fig. 1 are indeed representative of a normally transitory
phase of the osmotic hemolytic mechanism rather than of an
artifact. Specifically, our experiments show the following.

1) <u>The RPS spectra of neonate ghosts have characteristics
consistent with the existence of a large local lesion</u>. A
 dramatic and nearly instantaneously induced (apparent) size
drop of neonate ghosts, compared to critically swollen intact
cells, is clearly seen in Fig. 2A(a). The observed response
can be likened to the reaction of an uncapped tube of toothpaste
when squeezed. Considerable   "forces" are known theoretically
and experimentally to be exerted on intact cells flowing through

a small aperture (2, 14, 15, 16, 17). This interpretation
is contrary to the "intrinsic size reduction and reswelling"
concept previously hypothesized by others, based on the
limited data obtainable from the sizing techniques then
available (18).

2) Cells exposed to prehemolytic, hypotonic solutions
(i.e., between 150 and 300 mosm in buffered saline) contain-
ing 0.25% glut. show no evidence of adverse reaction to glut.
That is, they display no microscopically observable lesion
or other evidence of hemolysis, and no significant differences
in swelling or surface morphology from cells not treated with
glut.

3) In the process of being "captured in the act" by glut.,
cells swell to a volume equal to the maximal critical volume
achieved by native cells, prior to hemolysis as demonstrated
by RPS (19), as well as phase contrast microscopy (using the
methods given in Figs. 1 and 2A). That is, the membranes of
cells hemolyzing in the presence of glut. have not been ostensibly
weakened, and the cells' normal influx of osmotically driven
water has not been impeded.

4) Restored ghosts treated with 0.25% glut. and analyzed
by the RPS methods shown in Fig. 2B retain the ability to respond
osmotically, and remain highly permeable to water and relatively
impermeable to sodium ions. (Restored ghosts are the spontaneously
self-repaired membranes derived from red blood cells that have
lost most of their hemoglobin during hemolysis and have been
subsequently returned to isotonicity (20).) This behavior

further supports the supposition that 0.25% glut. is a mild agent - not chemically corrosive - with respect to the cell membrane.

Ghost Properties. When the neonate ghosts are treated with glut., the "squeezing" forces encountered while flowing through the device aperture become, as expected, much less effective in deformation and volume expulsion of intracellular contents (compare solid and dashed curves, Fig. 2A(b)). For the unfixed, neonate ghosts, if the stressing forces are themselves reduced, there is also a proportionate reduction in deformation. This is illustrated by a comparison of the curves in Fig. 2A (a and a') for which the ratio of flow rates is 5:1.

The opening of the neonate ghost is transient; that is, with increasing time, the lesion effectively repairs itself. This is evidenced in RPS by the reduced deformability of ghosts responding to the challenge of flow stress, as they recover (see Fig. 2A(a-d)). The fraction of lysed cells (ghosts) in the whole population is determined from the relative number of counts ("cells") in the two RPS subpopulations. This fraction is dependent upon the time elapsed since exposure to the lytic solution (e.g., compare Fig. 2A (a) with 2A (b)) as well as on the condition of the cells themselves. This kind of subpopulation analysis allows us to detect small differences in rates of hemolysis between different cell samples.

It is reasonable to interpret the increasing apparent ghost volume as a measure of the effective rate and strength

of the mechanical repair of the membrane lesion. This repair appears to commence no later than 20 to 30 sec after the lytic event. The "mechanical strength" then increases in a nearly linear fashion until it reaches a relative plateau of recovery. With a reduction in external stress (flow rate), an increase in the rate of progression to the plateau state is found. For example, at 20% of the normal flow rate, about 150 sec are required to plateau as opposed to 400 sec at the normal flow (0.008 ml/sec). In sum, the effective mechanical strength of the repairing lesion is flow stress and time dependent.

Although the repairing lesion is mechanically vulnerable for at least 400 sec, the effective osmotic barrier of the neonate ghosts re-establishes much more quickly, i.e., within 10 to 60 sec following the lytic event. This is demonstrated by a comparison of the volume response of the ghosts exposed at any given time to changes in external salt concentration. Both young ghosts (10 to 60 sec) and older ghosts (10 min) behave as nearly ideal osmometers (as determined by later RPS volume measurements) as long as they are not mechanically stressed until late in the recovery process.

The RPS spectra of ghosts allowed to repair for 10 min and then restored to an isotonic medium are seen in Fig. 2B. The dependences of the apparent volume distribution of the restored ghosts on changes in flow rate and on glutaraldehyde fixation are very similar to those of isotonically suspended

intact cells. In particular, with normal flow, unfixed ghosts (Fig. 2B(a)) and native RBCs show comparable bimodal distributions (21). Furthermore, this distribution becomes unimodal with reduced flow rate or upon rigidification with glut. (We have previously related the RPS bimodality characteristics to deformability of intact RBCs (2). This is to be distinguished from the "volume expulsion-deformation" behavior discussed above and illustrated in Fig. 2A(a)). At first this seems remarkable, considering that an intact cell containing about 34% hemoglobin has an internal viscosity of about 7 centipoise (cp) while that of a restored ghost is about 1 cp (22). Despite this approximately seven-fold difference in internal viscosity, the "bimodality-deformation" of the restored ghosts is nearly the same as that of intact cells, as indicated by the similarity of the spectra (23). It has been reported elsewhere that for measured RBC deformability and deformation inferred from viscometry, the internal viscosity is the dominant factor (24). Our results suggest that for the short-time (about 15μ sec), high-stress (2000 to 4000 dynes/cm^2) challenge, it is the intrinsic membrane deformability that dominates the RPS bimodality response.

In. further investigations of the posthemolytic repair processes, we have used RPS to follow the response of isotonically restored ghosts to subsequent re-exposure to hypotonic solutions. We find that as the external salt concentration is reduced below the original lytic osmotic composition, the repaired ghosts reach a critical volume very nearly the same as the original intact-cell critical volume. The ensuing "neonate II" ghosts have a flow-induced, volume drop measured by RPS very similar

to that of the initial event seen in Fig. 2A(a). These
findings suggest that the process of hemolysis and the repair
of the initial lesion have occurred without significant change
in total membrane area compared to that of the original intact
cell. They also indicate that both the effective mechanism
and the size of the lesion in the rehemolysis are similar to
those in the original lytic process (25).

The above types of membrane-hemolysis investigations
are not limited to studies on normal cells or to RBCs alone.
Valuable information can be obtained in this manner, simply
and rapidly, relevant to a variety of other types of cell investi-
gations. These include the effects of aging, drugs and other
agents, and studies on pathologies involving cell-membrane
integrity and repair mechanisms.

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References and Notes

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10. P. Seeman, <u>Fed. Proc.</u> 33, 2116 (1974).

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12. Based on our microscopic measurements and in agreement with values reported by P. B. Canham and A. C. Burton, <u>Cir. Res.</u> 22, 405 (1968); and E. A. Evans and Y. C. Fung, <u>Microvas. Res.</u> 4, 335 (1972).

13. As an individual cell in a dilute suspension traverses a current limiting aperture-transducer, a single "resistive" pulse is generated. The height or magnitude of the pulse is commonly taken as proportional to the volume of the (non-conductive) cell, and hence is used as a measure of cell size (W.H. Coulter, <u>Proc. Nat. Electron. Conf.</u>

12, 1034 (1956); G. Brecher, E.F. Jakobek, M. A. Schneiderman, G. Z. Williams, P. J. Schmidt, <u>Ann. N.Y. Acad. Sci.</u> 99, 242 (1962). The pulse height is also dependent, in a complex way, on cell deformability and shape - factors which influence the preferred orientation and trajectory of each cell as it flows through the aperture. This is crucial to the pulse form generated, given the irregularity of electrical field and hydrodynamic flow associated with relatively short apertures (16, 17, 21). RPS spectra as well as Coulter "volume spectra" are cumulative pulse height distributions obtained from the passage of thousands of cells over controlled volume or time intervals. Unlike conventional Coulter sizing, however, RPS analysis utilizes controlled variations in flow rate and in glutaraldehyde rigidification to permit evaluation of deformability as well as optimal resolution of actual cell size distribution (2).

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18.   Weed and Bowdler proposed than an intrinsic volume loss occurred with hemolysis, followed by a subsequent reswelling resulting from colloidal osmotic effects of residual

internal hemoglobin. (R.I. Weed and A.J. Bowdler, <u>J. Clin.</u>

<u>Invest.</u> 6, 1137 (1966)). Seeman, <u>et al.</u> suggested a mechan-

ical "collapse" followed by reswelling or a temporary

"short-circuit" effect related to the hemolytic event,

based on <u>mean</u> volumes rather than subpopulation data (P.

Seeman, T. Sauks, W. Argent, W.O. Kwant, <u>Biochem. Biophys.</u>

<u>Acta</u> 183, 476 (1969).

19. $V_a = \gamma V_t$ where V_a is the apparent volume (peak channel),

V_t is the true volume, and γ is the theoretical shape

factor. Generally, the shape factor is taken as 1.5 for

a sphere, 1.2 for a biconcave disc, and 1.0 for an elon-

gated prolate ellipsoid having its major axis oriented

in the direction of the electric field (17, 18; E.C. Gregg

and K.D. Steidley, <u>Biophys. J.</u> 5, 393 (1965)). From our

RPS measurements, V_a for the critically swollen cell is

2.3 times as large as V_a for the initial, isotonic, native

cell. Using these shape-factor corrections, we estimate

V_t (critically swollen) to be 1.6 to 1.7 times V_t (iso-

tonic). This is consistent with our optically measured

critical volume of $150\,\mu^3$ compared with a normal RBC volume

of $90\,\mu^3$, which gives a comparable ratio, 1.67. We calculate

V_a (neonate ghost)/ V_a (critically swollen cell) to be

about 0.60 (based on subpopulation peak channels, such

as those seen in Fig. 2B(a)). A substantial volume expulsion-

deformation effect must underlie such a large (40%) apparent

volume decrease, given the <u>requirement</u> of such an expulsion

to effect a shape change for a sphere bounded by an inextensible

membrane (12).

20. J. F. Hoffman, M. Eden, J.S. Barr, R. Dedell, <u>J. Cell Comp. Physiol.</u> 51, 405 (1958); H. Bodemann and H. Passow, <u>J. Membrane Biol.</u> 8, 1 (1972).

21. We (2) and others (e.g., Shank <u>et al.</u>) have discussed the apparent bimodal size distributions observed for intact normal RBCs in short apertures (approximately 50μ) of approximately 50μ diameter (B.B. Shank, R.B. Adams, K.D. Steidley, J.R. Murphy, <u>Lab. Clin. Med.</u> 74, 63 (1969)).

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23. Oscilloscope observations of individual resistive pulse forms for restored ghosts indicate that these forms (and hence the ghost trajectories) are about the same as those for intact cells.

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25. The <u>rate</u> of repair of the rehemolyzed ghosts is, however, observed to be slower than that of the primary event.

26. Hypotonic solutions of standard Dulbecco PBS (R. Dulbecco and M. Vogt, <u>J. Exp. Med.</u> 99, 167 (1954)).

27. Supported in part by the U.S. Energy Research and Development Administration, and by the Biophysics Training Grant TO1-GM00829.

Figure Legends

<u>Fig. 1.</u>   <u>Views of hemolyzing red blood cells (RBC)</u>.   Human

RBCs were added to a glutaraldehyde-containing, hypotonic,

phosphate-buffered saline (PBS) (26) (0.1 ml packed cells mixed

with 10 ml of solution containing 9 ml of 107-mosm PBS, pH 7.3,

and 1 ml of 2.5% glutaraldehyde, E.M. grade, Polysciences,

Inc.).   The 0.25% final concentration of glutaraldehyde was

chosen because it sufficiently cross-linked the hemoglobin

so that a "cast-molding" of the blowout lesion could be seen

yet it was sufficiently mild for the membrane to remain osmoti-

cally responsive.   (If the glut. fixative is added <u>subsequent</u>

to exposure to the hemolytic solution, even as little as 1

sec later, a smooth symmetrical shape without visible lesion

is observed.)

   (a)   <u>Phase-contrast photomicrographs (about X 1850)</u>, com-

posite of segments from three fields of hemolyzing RBCs, chosen

to display different aspects of the hemolytic lesion.   A single,

localized, circular rupture is seen for all hemolyzing cells.

Essentially all cells in the fields observed were in the erup-

ting condition.   The RPS-measured volume distribution of these

lysing cells is the same as that for maximally swollen intact

cells; their mean volume estimated from optical measurements

of diameters is about $150\,\mu^3$.

   (b)   Composite of three SEM fields taken from similar sam-

ples, selected to include some intact cells.   Considerable

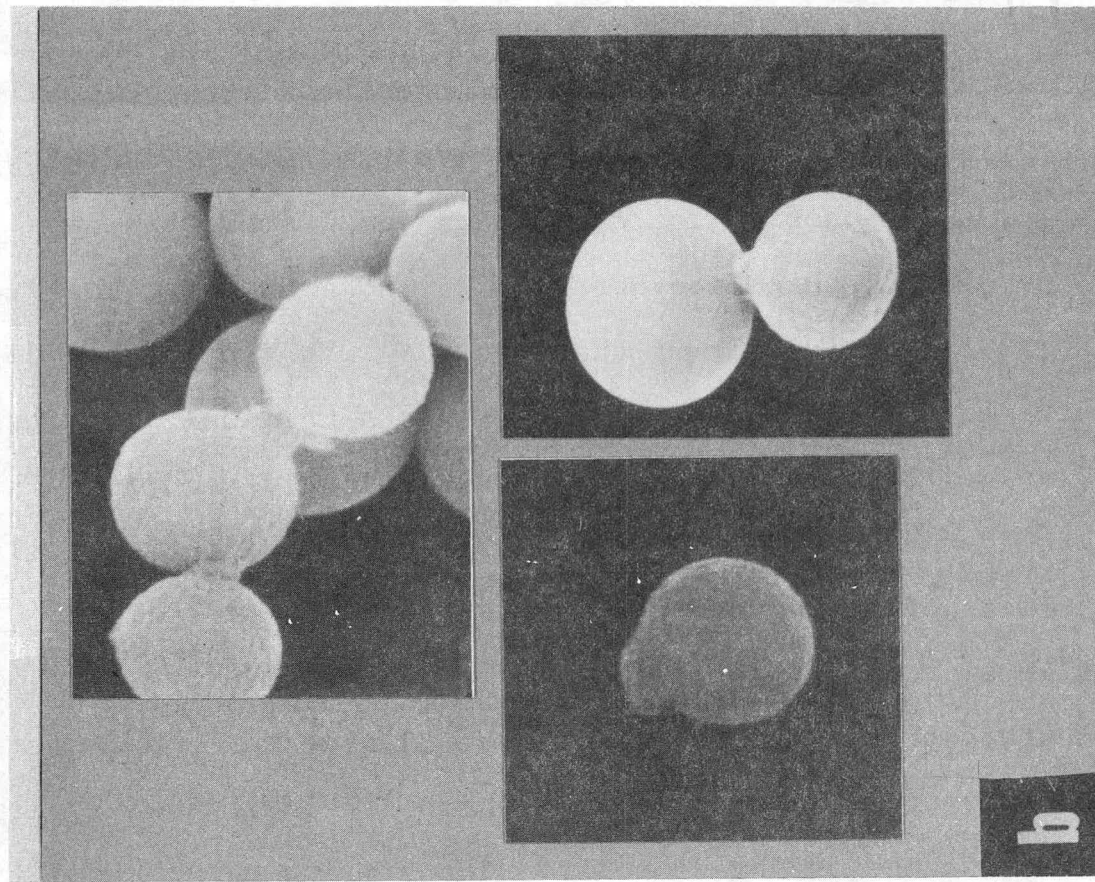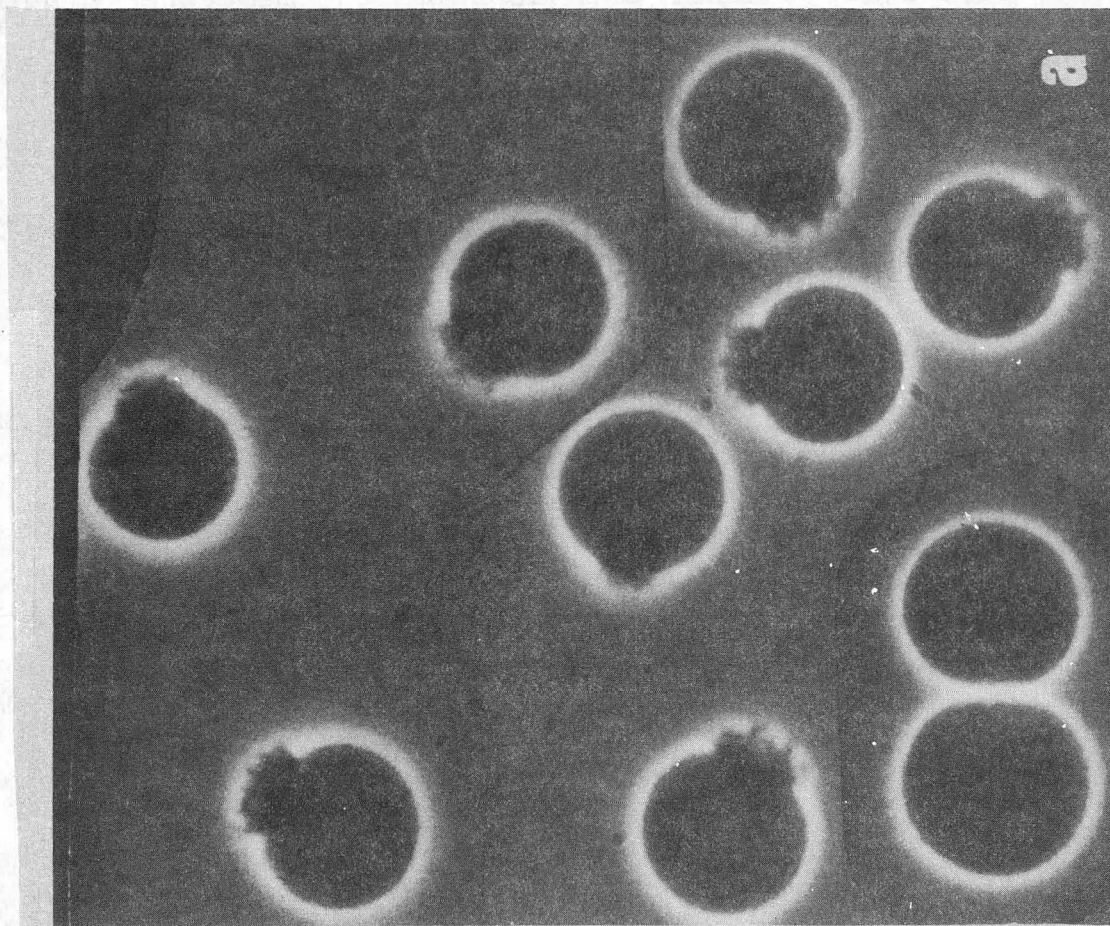shrinkage occurs during the dehydration and critical-point

drying, but the localized rupture is clearly preserved. The "wrinkled skin" appearance of the hemolyzing cells can be contrasted with the "taut skin" appearance of the critically swollen intact cells, attributable to the greater (artifactual) shrinkage of the ghosts.

Fig. 2A. Kinetics of the hemolytic process. Human RBCs mixed at time 0 with hypotonic PBS (140 mosm, pH 7.3) (26) were followed by RPS in a continuous sequence of 5-sec measurement intervals under automatic program control (5 x 10^5 cells/ml; 200 μ amp current; 48-micron-diameter orifice, Particle Data, Inc., Elmhurst, Ill; Digital Equipment Corp. PDP 8-I computer). The dynamic behavior of the cell-ghost subpopulations that occurs at a normal flow rate (0.008 ml/sec, about 4 m/sec mean velocity) is shown in selected spectra (a - d). The end-interval times (in seconds) and peak positions are indicated for each of the 64 channel spectra. The strong dependence of the spectra on flow stress is evidenced by comparison with a repeat preparation (a' - d') using reduced sample flow (0.0024 ml/sec). In separate experiments, the intrinsic cell-membrane deformability was greatly reduced at t = 60 sec by rapid addition of glutaraldehyde (final concentration 0.25%). Dashed curves in b and b' represent fixed cells corresponding to the native cells (solid curves in b and b'). Microscopic counts on these sample suspensions and on others prepared with compositions ranging from 0 to 100% in ghosts confirm that the "smaller" of the two (unfixed) subpopulations, as measured by RPS, corresponds to ghosts (most

distinct in a - c). The ghost population is seen to blend

upward into the intact cell population with increasing time

(e.g., series a - d), reduced flow rate (i.e., reduced shear

stress: compare a - a', b - b', etc), or reduced deformability

from glutaraldehyde treatment (i.e., b vs. dashed b). These

and other results strongly suggest that the initial "blowout"

lesion in the membrane renders the cell highly deformable,

especially for early times before appreciable repair has occurred

(see text). (Accordingly, it is the dominant role played by

the increased deformability of the "young" or "neonate" ghost

that makes its size unnaturally small.) The rate of "growth"

of the ghosts toward the intact cell peak is interpreted as

the rate of repair of the hemolytic lesion (e.g., compare peak

channels in series a - d). Also to be noted here

and in Fig. 2B is the intrinsic flow-rate dependence of apparent

size (peak position) for relatively less deformable objects

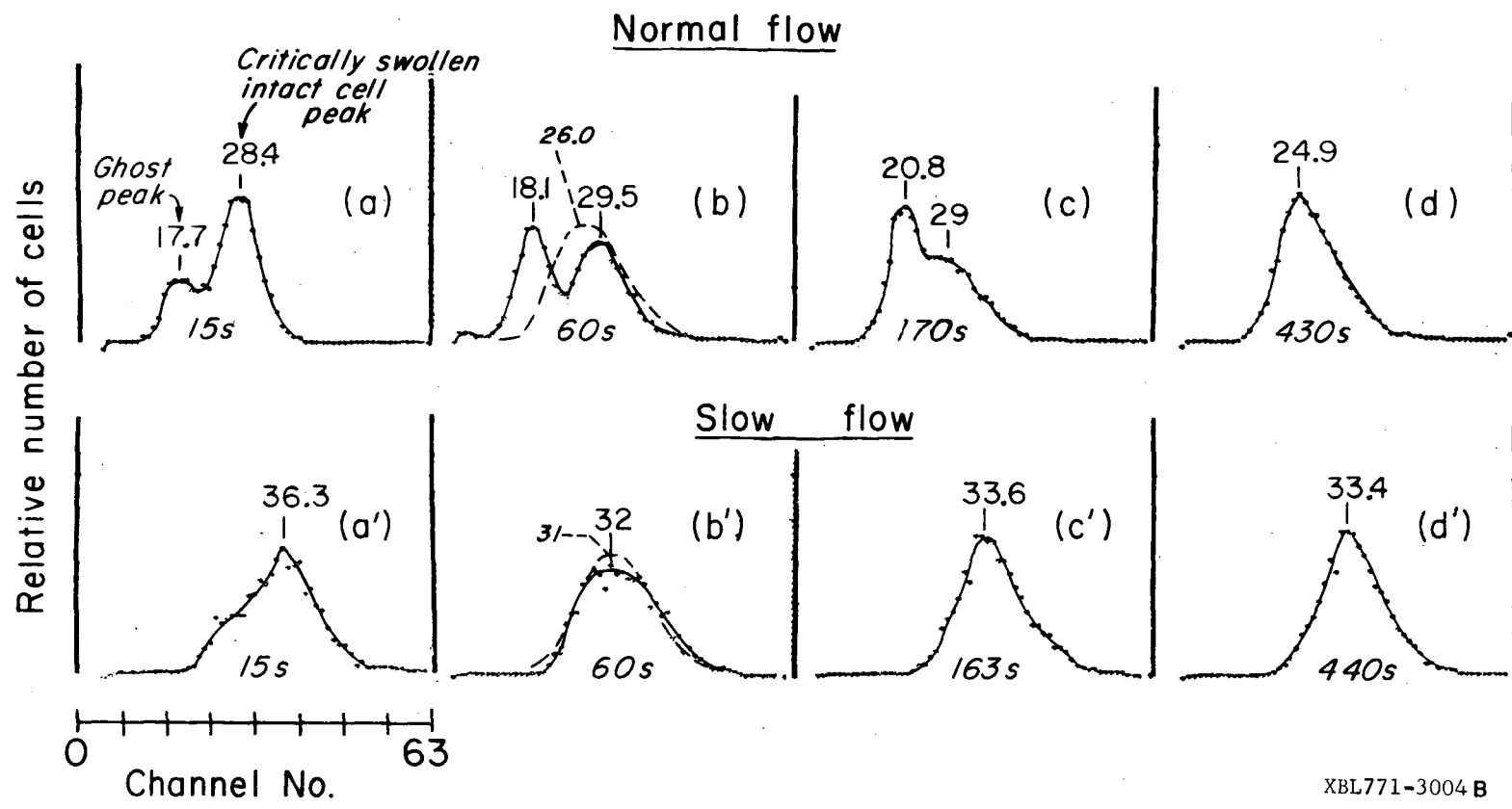(e.g., 2A: d - d', dashed b - dashed b') as previously described

(2).

Fig. 2B. Rheological properties of restored ghosts. Normal

human RBCs were added to 120 mosm PBS (26). After 10 min at

$22^{\circ}C$, the resultant suspension of ghosts was adjusted to 300

mosm by adding concentrated PBS, and the four basic RPS spectra

were run as shown (2) (200 μ amp   current; gain rela-

tive to spectra of Fig. 2A: 1.67x). The peak and mean channel

values are indicated for each of the 64-channel spectra. These

ghosts behave as intact native cells with respect to the following

characteristics: (i) the cumulative RPS spectral distri-

bution is bimodal at normal flow but transforms to unimodal
at slow flow (i.e.,a and a', respectively); (ii) the <u>individual</u>
pulse forms for ghosts (not shown) are very similar in rise
time and overall shape and duration to those of intact native
cells (23); (iii) fixation of the ghosts (0.25% glutaraldehyde)
converts the bimodal distribution (a) to a unimodal distribu-
tion (a'). The above phenomena have previously been associated
with the deformability and deformation of native intact cells
(2). (One notable difference is that fixed ghosts, unlike
fixed RBCs, retain osmotic activity.) The present results
demonstrate that restored ghosts have rheological responses in
flowing through small capillaries which are in some ways very
similar to those of intact cells. The similar RPS bimodality
behavior allows us to conclude that it is the membrane rather
than the internal viscosity of the cell which dominates this
type of deformability property of RBCs.
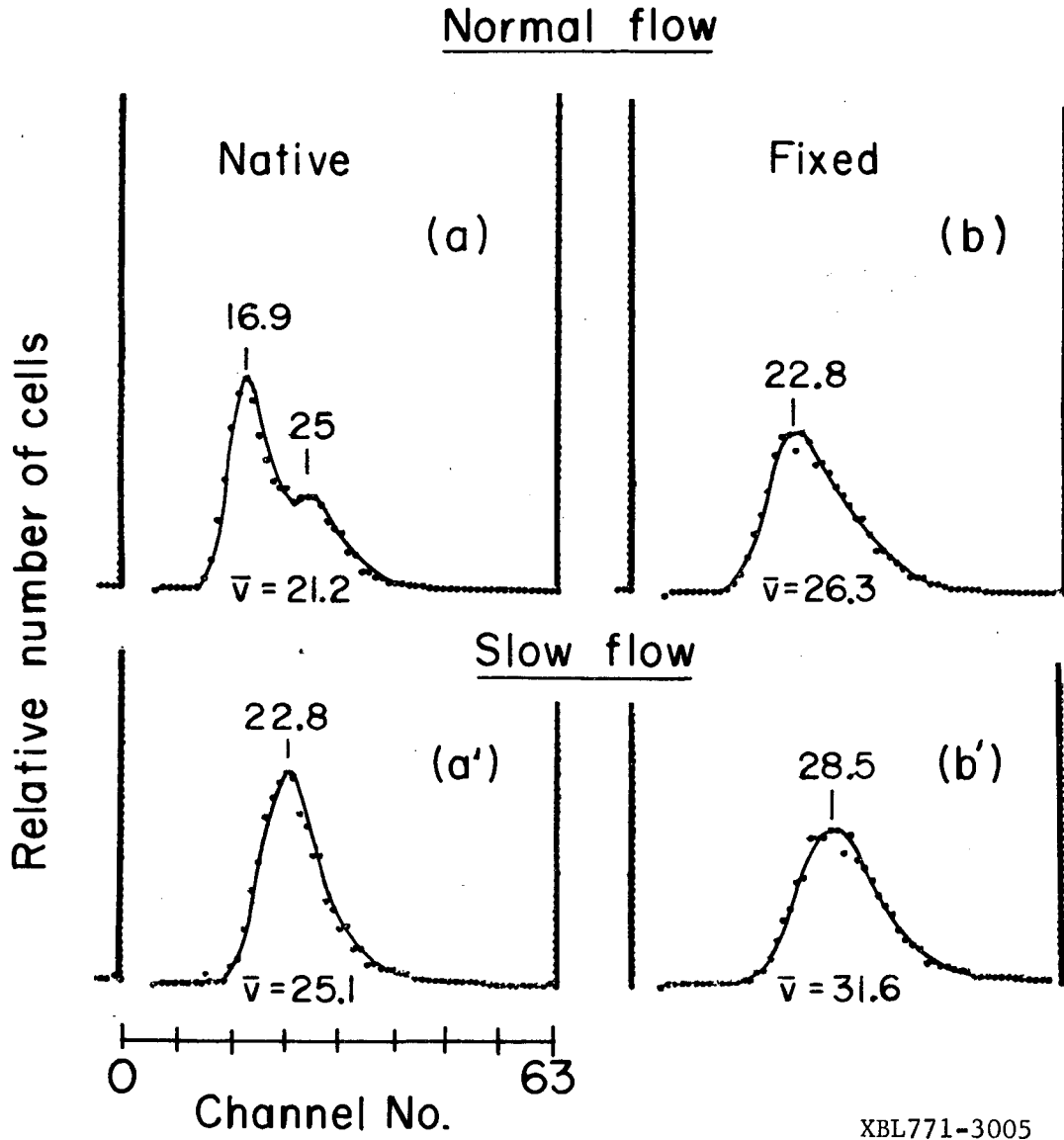
Fig. 1

Fig. 2A

XBL771-3004 **B**

Fig. 2B

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