6. Snails were fed daily with lettuce supplemented with vitamins. Water was freely available at all times.
7. G. Woolfe and A. D. Macdonald, *J. Pharmacol. Exp. Ther.* **80**, 300 (1944).
8. The hot plate was made by Technilab Instruments, Inc., Dequannock, N.J.
9. Terrestrial snail activity has been shown to be affected by hydration state [L. H. Smith, Jr., *Physiol. Zool.* **54**, 407 (1981)]. In our study, all snails tested were allowed to hydrate fully in sealed containers with a saturated atmosphere and water in the bottom. Hydrated individuals had a fully extended foot and were actively moving in the container.
10. Control experiments were conducted with the hot plate at lower temperatures (20° to 35°C). In addition, snail activity was monitored in thermal gradients and in field situations (B. Goodson, in preparation). Under normal thermal conditions this foot-lifting behavior was absent. *Cepaea* can display a "rearing" behavior in response to tactile stimulation of the head tentacles. This rearing involves an elevation of the entire body, including the shell, and is similar to the behaviors described for *Helix* [R. A. Everett, R. S. Ostfeld, W. J. Davis, *Z. Tierpsychol.* **59**, 109 (1982)].
11. Physiological saline consisted of NaCl, 80 m*M*; KCl, 5 m*M*; $CaCl_2$, 7 m*M*; $MgCl_2$, 5 m*M*; and tris buffer, 5 m*M*; final *p*H 7.8 [K. Meng, *Naturwissenschaften* **19**, 470 (1958)].
12. The snail body mass without a shell was 0.70 to 1.30 g.
13. R. W. Smith, M. Hirst, C. W. Gowdey, *Arch. Int. Pharmacodyn. Ther.* **221**, 266 (1976).
14. J. C. Sawynok, J. C. Pinsky, F. S. LaBella, *Life Sci.* **25**, 1621 (1979).
15. F. C. Tulmay, S. B. Sparker, A. G. Takenou, *Eur. J. Pharmacol.* **33**, 65 (1975); A. Goldstein, G. T. Pryor, L. S. Otis, F. Larsen, *Life Sci.* **18**, 599 (1976); A. Pert and M. Walter, *ibid.* **19**, 1023 (1976); R. J. Bodnar, D. D. Kelly, A. Spiaggig, C. Ehrenberg, M. Glusman, *Pharmacol. Biochem. Behav.* **8**, 667 (1978).
16. G. J. Dockray, C. Valliant, R. G. Williams, R. J. Gayton, N. N. Osborne, *Peptides* **2** (Suppl.) 25 (1981); R. J. Gayton, *Nature (London)* **298**, 275 (1982).
17. We thank B. Goodson for care of the snails, I. Craig for photographic skills, and Endo Laboratories, Inc., for a generous supply of naloxone hydrochloride. Supported by Natural Sciences and Engineering Research Council of Canada grant S222A1 (to M.K.) and Medical Research Council grant MA-7278 (to M.H.).

13 August 1982; revised 21 December 1982

Mechanical Measurement of Red Cell Membrane Thickness

Abstract. *The thickness of intact human red cell membrane is measured by a light-microscope technique in which membrane material with a known surface area is extracted into a long, thin cylindrical strand. The radius of the strand is calculated from its known length and surface area. The minimum radius, obtained at high extraction velocities or large membrane tensions, is 55 angstroms. A collapsed membrane cylinder with a mean-mass radius of 55 angstroms would have a membrane thickness of 78 angstroms.*

Benjamin Franklin observed (*1*) that one teaspoon of oil (4.93 cm^3), when allowed to spread on Clapham pond, would cover about ½ acre (2.02×10^7 cm^2). If we assume that the volume of oil in Franklin's experiment remains constant during the spreading process, then we can easily calculate that the thickness of the oil film is 24 Å. Thus, a submicroscopic dimension is readily calculated from macroscopic measurements such as, in this case, surface area and volume. In this spirit, we have devised an experimental technique that permits calculation of red cell membrane thickness, using only the "principle of conservation of membrane surface area" and measurements made with a light microscope.

In our experiments we allow nearly spherical fresh human red cells suspended in a hypotonic phosphate-buffered solution (*p*H 7.4, 160 mos*M*, 0.05 to 0.08 g percent albumin) to settle and adhere to latex beads 2 μm in diameter, forming cell-bead pairs. Subsequently, we aspirate a portion of the cell membrane into a small glass pipette and capture the latex bead with another smaller glass pipette, thereby suspending the cell and bead between two pipettes (Fig. 1A). Movement of the bead and smaller pipette away from the cell results in the formation of a long, thin membrane filament or "tether" (*2*), observed as a faint shadow stretched between the cell body and the point of membrane attachment (Fig. 1, C and D). As we increase the aspiration pressure and thus the isotropic tension in the membrane (*3*), the cell becomes more spherical, and the tether shadow becomes fainter as the tether diameter decreases until it disappears from view (Fig. 1A). Reducing the tension results in elongation of the cell and reappearance of the tether. In Fig. 1B a schematic of the aspirated cell and formed tether shows a greatly magnified end-on view of the tether indicating the phospholipid bilayer membrane geometry.

Initially, it would seem impossible to measure in situ something as thin as a tether. However, the method of tether formation shown in Fig. 1 permits us to measure the tether radius as it is formed. In essence, a reservoir of membrane material is sucked into the pipette. As we pull the tether from the cell body, the membrane material in the pipette is reduced. Since the aspiration pressure is held constant during tether formation (or reabsorption), this process occurs at constant membrane surface area and constant cell volume (*4*). For the sake of illustration, we write that the decrease in membrane material within the pipette is balanced by an increase in membrane material in the tether:

$$-2\pi R_p dL_p \approx 2\pi R_t dL_t \qquad (1)$$

where R_p is the (measurable) radius of the pipette, $-dL_p$ is the (measurable) decrease in length of the aspirated por-

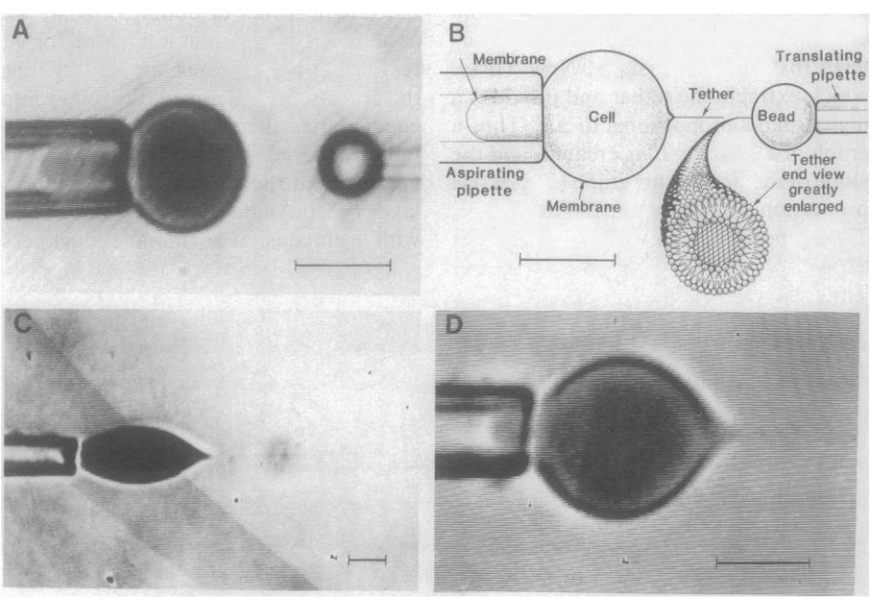

Fig. 1. Photographs taken from a video monitor of tethered red cells. (A) Red cell aspirated into the larger pipette and latex bead held by the smaller pipette. The (invisible) tether is stretched between cell and bead. (B) Schematic of (A) showing a greatly magnified end-on view indicating the phospholipid bilayer membrane geometry. (C) Flaccid red cell with a relatively thick tether. (D) Partially sphered cell. Note how the tether pulls on the cell body. An increase in the aspiration pressure causes the cell to assume a nearly spherical shape (A) and aspirates more membrane material into the pipette. However, the tether still produces a small amount of cell distortion at a "point" on the cell membrane opposite the point of aspiration. Scale bars, 4 μm.

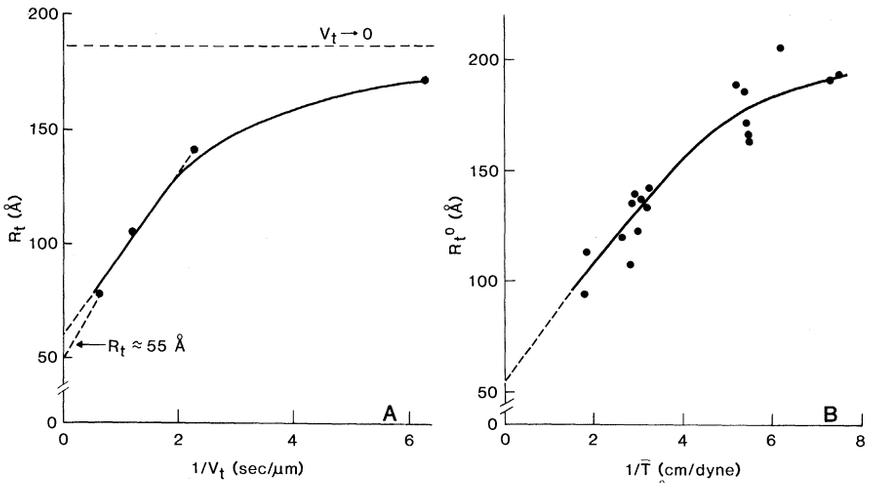

Fig. 2. Extrapolation of tether radius to a minimum value of about 55 Å. (A) Extrapolation as $1/V_t \rightarrow 0$ at a constant isotropic tension $\bar{T}$ of 0.19 dyne/cm. (B) Extrapolation as $1/\bar{T} \rightarrow 0$ at zero velocity.

tion of the membrane within the pipette, and dL_t is the (measurable) increase in tether length. This relation allows us to calculate a value for the tether radius R_t:

$$R_t = R_p \left(- \frac{dL_p}{dL_t} \right) \quad (2)$$

Imposition of the constant-volume constraint results in only a slight modification of Eq. 2:

$$R_t = \left(1 - \frac{R_p}{R_c} \right) R_p \left(- \frac{dL_p}{dL_t} \right) \quad (3)$$

where R_c is the maximum radius of the cell (*4*). Typically, $R_p/R_c = \frac{1}{3}$ and thus the "correction term" in Eq. 3 has a value of about 0.7.

Movement of the membrane "tongue" down the inside of the pipette ($-\Delta L_p$) can be measured with a high degree of precision. This tracking of the tongue gives accuracies on the order of 10 percent for overall length changes of 1 to 2 μm. The change in length of the tether, ΔL_t, is produced and measured with an electromechanical linear translator (Burleigh Instruments, Fishers, New York), which extracts the tether and provides a digital signal proportional to ΔL_t. Thus a graph of L_p versus L_t is created, and the slope of the line on this graph (dL_p/dL_t) is proportional to the tether radius (Eq. 3). The slope is constant, which indicates that the tether radius remains constant during tether formation (*4*). Although the overall slope of this line can be measured with an accuracy of about 10 percent, the calculated value for R_t from Eq. 3 may be no more accurate than ±30 percent because of an estimated 20 percent error in the measurement of the pipette diameter caused by optical distortion at the fluid-pipette interface (Fig. 1).

As we repeatedly extract the same tether from a given cell at different velocities, we observe a decrease in the tether radius R_t as we increase the extraction velocity V_t (Fig. 2A). Also, R_t increases when we increase the absorption velocity. In addition, when we obtain an extrapolated or interpolated value at zero velocity (let $R_t = R_t^0$ when $V_t = 0$), we observe R_t^0 decrease as we increase the aspiration pressure (isotropic tension $\bar{T}$ in the membrane) as shown in Fig. 2B (*5*). Thus the linear extrapolations shown in Fig. 2 indicate a minimum value for the tether radius of about 55 Å. If we assume that the minimum value for R_t represents a mean-mass radius dividing the cross-sectional area of the tether into equal parts, then the thickness of the tether material is 78 Å (*6*). This value is greater than the thickness of a lipid bilayer (~ 50 Å) and is in good agreement with estimates of membrane thickness made from x-ray and neutron scattering density profiles of suitably prepared, packed red blood cell membranes (*7*). These simple mechanical experiments, however, provide a direct measurement of the membrane thickness of a single, intact human red cell.

R. M. Hochmuth
E. A. Evans*
H. C. Wiles
J. T. McCown†
Department of Biomedical Engineering,
Duke University,
Durham, North Carolina 27706

References and Notes

1. B. Franklin, in *The Ingenious Dr. Franklin: Selected Scientific Letters of Benjamin Franklin*, N. G. Goodman, Ed. (Univ. of Pennsylvania Press, Philadelphia, 1931), p. 190.
2. R. M. Hochmuth, N. Mohandas, P. L. Blackshear, Jr., *Biophys. J.* **13**, 747 (1973). In our original experiments, tethers were extracted from flaccid rather than spherical red cells by subjecting the adherent cells to a fluid shear force. The diameter of the tether decreased as the force exerted on the tether was increased (by increasing the fluid flow). A rapid increase in the force caused the formation of small globules (presumably hemoglobin), which would move up the tether and subsequently disappear into the cell body. A decrease in the force permitted the tether to be reabsorbed by the cell. Thus we concluded that the tether is a hollow elastic fiber formed from membrane material.
3. R. P. Rand, *Biophys. J.* **4**, 303 (1964); E. A. Evans, R. Waugh, L. Melnik, *ibid.* **16**, 585 (1976). For a spherical cell, both Rand and Evans *et al.* showed that the isotropic tension $\bar{T}$ in the membrane, and the pipette aspiration pressure ΔP, are related by
$$\bar{T} = \frac{(\Delta P) R_p / 2}{1 - R_p/R_c}$$
where R_p is the radius of the pipette and R_c is the radius of the cell.
4. R. M. Hochmuth and E. A. Evans, *Biophys. J.* **39**, 71 (1982); R. M. Hochmuth, H. C. Wiles, E. A. Evans, J. T. McCown, *ibid.*, p. 83.
5. An interpolated value for the tether radius at zero velocity comes from the measurement of tether radii at both positive (extraction) and negative (absorption) tether velocities.
6. Consider the tether as a collapsed cylinder with the minimum value for R_t representing the mean-mass radius of this collapsed cylinder. Then, if we neglect local variation in density, the cross-sectional areas of the membrane material on either side of R_t must be equal:
$$\pi R_t^2 = \pi h^2 - \pi R_t^2$$
where h represents the (membrane) thickness of the collapsed cylinder, that is, the distance from the center of the cylinder to the outer surface. Thus, $h = \sqrt{2}\, R_t = 78$ Å.
7. L. McCaughan and S. Krimm, *Science* **207**, 1481 (1980).
8. This work is supported by NIH grant HL 23728.
* Present address: Department of Pathology, University of British Columbia, Vancouver, B.C., Canada V6T 1W5
† Present address: Lever Brothers Research Center, Edgewater, N.J. 07020.

15 October 1982