# Tensile strength and dilatational elasticity of giant sarcolemmal vesicles shed from rabbit muscle

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1. Mechanical properties of the surface membrane of skeletal muscle were determined on sarcolemmal vesicles (mean diameter, 71 $\mu$m) shed by rabbit psoas muscle swelling in 140 mM KCl containing collagenase.

2. Vesicles were stressed by partial aspiration into parallel bore pipettes. The isotropic membrane tension so created caused an increase in membrane area which expresses itself in an elongation of the vesicle projection into the pipette.

3. For individual vesicles, a linear relationship between membrane tension and membrane area increase was found up to the point when the vesicle burst, i.e. sarcolemmal vesicles behaved as perfectly elastic structures.

4. The maximum tension sarcolemmal vesicles could sustain before bursting was $12{\cdot}4 \pm 0{\cdot}2$ mN m$^{-1}$ (median $\pm$ 95% confidence interval), and the corresponding fractional increase in membrane area was $0{\cdot}026 \pm 0{\cdot}005$ (median $\pm$ 95% confidence interval). The elastic modulus of area expansion was $490 \pm 88$ mN m$^{-1}$ (mean $\pm$ S.D.).

5. In conformity with cited comparable work on red blood cells and artificial lipid vesicles, the strength and area elasticity of the skeletal muscle membrane are considered properties of the fluid lipid matrix of the membrane and of the degree to which the bilayer is perturbed by lipid–protein interaction.

The cell surface membrane as a permeability barrier and as a site of electrical potential has been studied exhaustively. By contrast, the mechanical properties of the cell membrane have scarcely been explored. The exception is the red blood cell membrane whose strength and elasticity have been determined by pipette aspiration (Rand, 1964; Evans, Waugh & Melnik, 1976; Waugh & Evans, 1979). In the present work, this technique has been adopted to study the mechanical properties of the surface membrane of mammalian skeletal muscle as presented in the form of sarcolemmal vesicles.

## Formation and properties of sarcolemmal vesicles

Bowman (1840) first described how the surface of a muscle fibre placed into water lifts off to form large blebs. A similar process was observed by Hogue (1919) in cultured cardiac tissue cells in hypotonic solution. With the phase microscope, Zollinger (1947) noted the formation of blebs on kidney, liver and other types of mechanically dispersed cells, and he described how such blisters become detached to form vesicles. Membrane blebbing has become a commonplace observation with the widespread use of enzymatically dispersed cells. It is generally regarded as indicative of a cell in less than perfect condition.

That skeletal muscle may form blebs and vesicles was rediscovered by Standen, Stanfield, Ward & Wilson (1984) on frog muscle. In order to clean the membrane for patch clamp experiments they depolarized the muscle in a high-$K^+$ solution and then treated it with collagenase and protease in sequence. They used 140 mM KCl as the depolarizing solution and so caused the fibres to swell (Boyle & Conway, 1941). The combined effect of fibre swelling and enzyme treatment was a crop of membrane vesicles with a median diameter of 80 $\mu$m. Burton, Dörstelmann & Hutter (1987) developed the simple procedure for the production of vesicles from mammalian muscle fibres which is used in the present work. Vesicles can also be produced without the use of enzymes and swelling solutions by stretching isolated single muscle fibres five to eight times their resting length (Stein & Palade, 1989). In such treatment the submembrane cytoskeleton is probably damaged by over-extension, so that the membrane becomes uncoupled from the rest of the fibre. Some kind of disruption of normal membrane anchoring presumably occurs also in enzymatically treated swelling fibres to cause floatation of the membrane. The vesiculation commonly observed in a proportion of enzymatically dispersed liver, cardiac or similar small cells, is usually attributed to degradation of the submembrane cytoskeleton

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by proteases activated by a rise in intracellular $Ca^{2+}$ (Nicotera, Hartzell, Davis & Orrenius, 1986).

As vesicles bud off, they enclose cytoplasmic ground substance, glycogen granules etc., but not organelles. Vesicles have been shown to contain the cytosolic enzyme creatine phosphokinase in amounts equal to intact muscle fibres, when expressed in terms of total cytosolic protein (M. J. Jackson & O. F. Hutter, unpublished observations). These findings clearly imply that the vesicle membrane remains in normal outside-out configuration. Electron micrographs of muscle fibres which have undergone vesiculation show that the transverse tubules and internal membranes remain intact. Heavily vesiculated fibres are denuded of outside membrane (D. L. Bovell, I. Montgomery & O. F. Hutter, unpublished observations). In view of their large surface area, the formation of vesicles must involve the disconnection of many transverse tubules from the surface membrane and the resealing of the points of rupture. The possibility that some membrane from the upper part of the transverse tubule is incorporated into the vesicle therefore cannot be ruled out. By way of further characterization of sarcolemmal vesicles as composed of cell surface membrane, they have been shown to contain $Na^+$–$K^+$-ATPase but not SR $Ca^{2+}$-ATPase (Zubrzycka-Gaarn *et al.* 1991). The lactate transport system remains operational in sarcolemmal vesicles with kinetic parameters as for intact muscle (Juel, 1991).

Thanks to the use of collagenase, sarcolemmal vesicles are devoid of basement membrane and so seal readily to patch pipettes. Advantage of this has been taken to study the delayed rectifyer $K^+$ channel (Standen, Stanfield & Ward, 1985) and the inwardly rectifying $K^+$ channel (Burton & Hutter, 1990). The behaviour of these channels in response to membrane depolarization provides additional evidence of the normal orientation of the vesicle membrane. Besides, patch clamp experiments can give information on the ionic composition of sarcolemmal vesicles. That the single channel conductance of the inwardly rectifying $K^+$ channel is smaller for outward than for inward current (Burton, Dörstelmann & Hutter, 1988; Burton & Hutter, 1990) connotes that sarcolemmal vesicles contain enough $Mg^{2+}$ to block that channel (Vandenberg, 1987). Similarly, the largely closed state of $K_{ATP}$ channels in vesicle-attached patches signifies that vesicles contain enough ATP to keep these channels inactive. High conductance $K_{Ca}$ channels are readily found in inside-out patches exposed to $Ca^{2+}$ containing solutions (Burton *et al.* 1988); but in attached patches these channels open only on strong depolarization, which signifies (Pallotta, Magleby & Barrett, 1981) that the concentration of $Ca^{2+}$ in vesicles is submicromolar, i.e. near the levels found in normal resting muscle fibres.

In summary then, sarcolemmal vesicles contain cytosol enveloped by plasmalemma. In all so far tested respects, the plasmalemma has retained its normal functional characteristics, despite its divestment of basement membrane and such disruption of the submembrane cytoskeleton as may be inevitable in vesicle formation. Sarcolemmal vesicles may therefore serve for measuring otherwise inaccessible mechanical properties of the muscle surface membrane.

**The pipette aspiration technique**

The aspiration method for studying the mechanical properties of the cell surface was introduced by Mitchison & Swann (1954), working on sea urchin eggs. Rand & Burton (1964) later applied this technique to red blood cells. Using micropipettes of about 2 $\mu$m diameter, they found that discoid or partly swollen red cells are at first easily pulled into the pipette. But when the part of the cell remaining outside the pipette became the portion of a sphere, the membrane resisted the increase in area which would be necessary, at constant cell volume, to draw more of the cell into the pipette. Rand (1964) showed that the stressed membrane will then burst at tensions of 10–20 mN $m^{-1}$. With the recognition that the red cell membrane is a composite structure consisting of a fluid lipid bilayer and proteins, some of which are linked to a filamentous submembrane cytoskeleton (Singer, 1974), it was realized that different material properties are being measured at different stages of a pipette aspiration experiment (Skalak, Tozeren, Zarda & Chien, 1973; Evans, 1973). So long as the cell is flaccid and aspiration produces a change in shape without an increase in membrane area, the elasticity of the submembrane skeleton determines the shear elasticity of the cell. By contrast, the resistance of a rounded-up cell to area dilatation is attributable to the strong cohesive force in lipid bilayers which arises from the hydrophobic effect (Tanford, 1973). By refining the aspiration technique, Evans and his colleagues were able to quantify the disparity between membrane deformability in shear and in dilatation. Thus the shear elasticity of the red cell is characterized by a modulus in the order of $10^{-2}$ mN $m^{-1}$, whereas the modulus of area expansion is about $5 \times 10^2$ mN $m^{-1}$ (Evans *et al.* 1976; Evans & Waugh, 1977). Artificially made unilamellar vesicles, when subjected to pipette aspiration, yield moduli of area expansion from $1 \times 10^2$ to $10 \times 10^2$ mN $m^{-1}$ depending on phospholipid to cholesterol ratio (Evans & Needham, 1987), which emphasizes that membrane mechanical behaviour during area dilatation is largely an expression of the properties of the lipid bilayer.

In the experiments here to be described, the initial suction applied to a sarcolemmal vesicle was always adequate to render spherical the portion of the vesicle outside the pipette. Any subsequent increase in suction, and hence increase in membrane tension, therefore produced membrane dilatation, i.e. an increase in area per membrane molecule. The results of the present measurements therefore provide information primarily about the cohesiveness of the

lipid bilayer. However, owing to the high density of proteins in biological membranes, the mechanical properties of sarcolemmal vesicles measured here also reflect any perturbation of the lipid bilayer through its interaction with proteins. The mechanical properties of sarcolemmal vesicles prepared by a standard method are described in this paper. In the subsequent paper the effect of loading vesicles with $Ca^{2+}$ will be described (Nichol & Hutter, 1996).

## METHODS

### Preparation of sarcolemmal vesicles

Mammalian muscles of all kinds will yield sarcolemmal vesicles, but rabbit psoas muscle is particularly suitable on account of the length and size of its fibres and its relative freedom from interfascicular connective tissue and fat. New Zealand White rabbits, both male and female, were killed by cervical dislocation or lethal injection of pentabarbitone and then bled out. After laparotomy, the peritoneum and fat overlying the psoas muscle was removed manually. A 3–4 cm length from the belly of the muscle was then freed from its dorsal attachment by blunt dissection, excised and placed in cold (4 °C) 140 mM KCl with no added $Ca^{2+}$. When the cut muscle segment had relaxed, it was pinned out in a large petri dish under 140 mM KCl onto a layer of Sylgard. Strips of muscle were then separated and placed into 5 cm polystyrene dishes containing a layer of Sylgard for further dissection under 140 mM KCl at room temperature, the objective being to arrive at an array of fascicles thin enough to allow microscopic observation of vesiculation. The fascicles were kept lightly stretched by fine pins and retained the normal striation pattern of resting muscle throughout vesiculation. To minimize debris, any obviously crushed ends were trimmed off with a razor blade and washed away with fresh 140 mM KCl. Preparation of a set of dishes containing fascicles usually took about 30 min during which time the fibres gradually swelled. KCl (140 mM) was always buffered with 5 mM Hepes and titrated with KOH to pH 7·4 at room temperature. No correction was made for the slight increase in pH to be expected when the solution was cooled to store muscle, or for the slight decrease in pH to be expected when the temperature was raised during enzyme action.

To induce vesiculation the preparations were exposed to 140 mM KCl containing 100 U $ml^{-1}$ type Ia collagenase (Sigma) and the dishes placed on a hotplate at 34 °C for 10–15 min. Evaporation during incubation was minimized by covering the dishes with plastic lids lined with moistened filter paper. The final step in the procedure was the transfer of the lightly drained fascicles into new dishes containing a measured volume of enzyme-free KCl solution, and the release of vesicles trapped between the fibres by gently teasing apart the muscle, which was subsequently removed from the dish. Harvesting too early gave low yields because too few vesicles had yet formed; harvesting too late reduced the yield because too many vesicles had already been shed. With the enzyme solution used here, 10–15 min of incubation was usually best. At all events, periodic microscopic observation soon informed of the correct moment, and, in practice, the whole procedure was much simpler than its description suggests. In one experiment, designed to test whether muscle fibre type determines membrane mechanical properties, the above procedure was applied to rabbit soleus muscle with equal success.

Muscle segments kept for up to 2 h in cold 140 mM KCl solution before dissection yielded vesicles indistinguishable from those immediately prepared as above. However, delay in the excision of the muscle from the carcass prevented the formation of vesicles. Whether this is due to rapid autolysis of the membrane after death, possibly as a result of a rise in intracellular $Ca^{2+}$, or whether incipient rigor extends to the submembrane cytoskeleton and prevents its disruption remains unexplored.

Vesicles as produced above vary in their refractivity. These differences in refractive properties are likely to be related to differences in the density of cytosolic content of the vesicles, as visible in electron microscope sections of pelleted vesicles (D. L. Bovell, I. Montgomery & O. F. Hutter, unpublished). For the present experiments the more refractive vesicles between 30 and 120 mm diameter were used (mean, 71·0 ± 11·5 mm). Most of the mechanical measurements reported here were made on vesicles left in 140 mM KCl with no added $Ca^{2+}$. When the effect of $[Ca^{2+}]_o$ was to be tested the concentration used is given in the text.

### Manufacture of pipettes

Pipettes were pulled from borosilicate glass capillaries (1·5 mm o.d., 1·2 mm i.d.) using a two-stage electrode puller set to produce pipettes with a shank of uniform bore ranging from 9 to 25 $\mu$m diameter (mean, 19·3 $\mu$m) over a length of about 500 $\mu$m, before terminating in a tip of 1–2 $\mu$m open diameter. To break such pipettes cleanly towards the end of the uniform bore shank, the tip of the pipette, mounted on a micromanipulator, was brought into contact with a bead of solder glass ($T_{soft}$ = 570 °C) kept molten on a loop of resistance wire. Due to the much higher melting point of the pipette glass, the pipette remained rigid as molten solder glass flowed into it from the bead by capillary action. When the solder glass had reached the uniform bore shank, the heating current was switched off and, on cooling, the solder glass in the pipette solidified with the bead. Quick withdrawal of the pipette from the bead then usually resulted in a clean break at the interface between the hollow and the solder glass-filled portions (Evans *et al.* 1976; Zhelev, Needham & Hochmuth, 1994). Any jagged pipettes were rejected. Satisfactorily square-ended pipettes were finally bent to an angle of approximately 25–30 deg, at a point roughly 5 mm from the break, by reheating the glass under a loop of resistance wire. This compensated for the slope of the pipette holder and allowed the working end of the pipette to lie parallel to the bottom of the dish. Pipettes were half-filled with filtered 140 mM KCl by back-filling through a fine tube.

### Experimental apparatus

A top-focusing microscope (Micro-instruments Ltd, Witney, Oxford, UK) fitted with long working distance ×4 and ×40 objectives was used. The eyepieces could be exchanged for a TV camera (Panasonic WV-BL200/B) coupled to a monitor and video recorder. The optical system was calibrated with a stage micrometer. The camera eyepiece magnified ×2·5 and was chosen so as to record at high gain a field of view of approximately 120 $\mu$m, enough to contain the largest vesicles used. The entire optical assembly could be raised away to allow easy access to the experimental dish held in a mechanical stage. With the highly refractory vesicles used, phase contrast optics were unnecessary. Slight defocusing of the condenser improved visualization of vesicles.

The aspiration pipette was held in a patch-clamp type holder with its suction tube coupled to a water manometer. Pipette pressure

was controlled by manual injection or withdrawal of air in the closed limb of the manometer with a 50 ml syringe. It could be measured visually from a scale with an accuracy of ± 1 $mmH_2O$ and was read aloud into the voice channel of the video recorder. Zero pressure was determined as the reversal point of flow in or out of the pipette in the dish.

### Aspiration technique

Before positioning the working end of the micropipette so as to lie level just above the bottom of the experimental dish, a slightly positive pressure was applied to keep debris away from it. The mechanical stage was manoeuvred so as to centre a settled vesicle in line with the axis and close to the mouth of the pipette. The pipette pressure was then reversed to between −0·5 and −1·0 $cmH_2O$. This gentle suction was enough to bring the vesicle towards the pipette and usually also to draw a projection into the latter. The pipette, with the vesicle attached to it, was next moved upwards away from the floor of the dish. In this way, any debris and erythrocytes lying at the bottom of the dish dropped out of the picture. The microscope was refocused first for measurement of the diameter of the spherical portion of the aspirated vesicle, and next for optimum visibility of the vesicle projection within the pipette. The suction pressure was then increased to 2 $cmH_2O$ and thereafter in steps of 2 $cmH_2O$ until the vesicle ruptured. At the end of the experiment the videotape and accompanying soundtrack were replayed and the pipette and vesicle diameters and the length of the projection into the pipette measured from the screen, starting with the readings at 2 $cmH_2O$. The experiments were done at room temperature (range, 19–25 °C).

### Analysis

In contrast to the patch clamp situation, in which much smaller-tipped, fire-polished pipettes are used, no seal forms between vesicle membrane and the walls of the aspiration pipette. Rather, the membrane tension created by aspiration is transmitted to the portion of the vesicle outside the pipette, i.e. an isotropic tension is set up. Following in the footsteps of Rand (1964) and Evans *et al.* (1976), the law of Laplace may therefore be applied to the hemispherical cap inside the pipette and to the spherical portion of the vesicle:

$$P_v - P_p = 4T/D_p, \quad (1)$$

$$P_v - P_o = 4T/D_v, \quad (2)$$

where $P_v$, $P_p$ and $P_o$ are the pressures in the vesicle, pipette and bathing solution, respectively. $D_p$ and $D_v$ are, respectively, the diameters of the pipette and spherical portion of the vesicle and $T$, the isotropic tension keeping the system in equilibrium (Fig. 1). As $D_p$ is smaller than $D_v$ the pressure difference across the cap needs to be larger than the pressure difference across the major portion of the vesicle, which is achieved by applying the aspiration pressure:

$$\Delta P = P_o - P_p.$$

$\Delta P$ is the measurable variable in the experimental situation and by subtraction of eqns (1) and (2) comes to:

$$\Delta P = 4T(1/D_p - 1/D_v) \quad (3a)$$

or

$$T = \Delta P \times D_p/4(1 - D_p/D_v). \quad (3b)$$

The membrane area of a vesicle aspirated into a pipette with light suction adequate to take up the membrane slack is given by

$$\pi D_v^2 - \pi D_p^2/4 + \pi D_p L, \quad (4)$$

where the first term is the area of a complete sphere of diameter equal to the aspired vesicle, the second term is the cross-sectional area of the pipette not covered by membrane, and the last term is the area of the projection, of length $L$, into the pipette. On increase in suction pressure and consequent dilatation of the membrane, the projection can grow longer. The attendant movement of the vesicle content into the pipette provides, at the same time, additional area for elongation of the projection. The increase in membrane area attributable to membrane dilatation under stress, $\Delta A$, will therefore be given by:

$$\Delta A = \pi D_p \Delta L - \pi(D_{v0}^2 - D_{v1}^2), \quad (5)$$

where $\Delta L$ is the increase in the length of the projection, $D_{v0}$ is the initial diameter of the vesicle aspirated with light suction and $D_{v1}$ the smaller diameter of the pressurized vesicle. As the membrane can suffer only a small area dilatation, the volume displacement into the pipette is too small for $D_{v1}$ to be measurably different from $D_{v0}$, but assuming constant total volume $D_{v1}$ will be given by:

$$D_{v1}^3 = D_{v0}^3\{1 - [(6D_p^2\Delta L)/4D_{v0}^3]\}. \quad (6)$$

A computer program which calculates this term and solves for $\Delta A$ by inserting it into eqn (5) has been written by Dr F. L. Burton and was used by Hutter, Burton & Bovell (1991). The programme also calculates $T$ from eqn (3b) and the best-fit slope of the stress–strain relation from which the origin of the $\Delta A$ axis was established by extrapolation, i.e. due allowance was made for the membrane area dilatation caused by the initial suction pressure of 2 $cmH_2O$.

The assumption that the volume of vesicle plus projection remains constant during aspiration needs to be justified, as the pressurization of vesicles should, in principle, cause water to leave the vesicle until an osmotic pressure is set up to oppose the

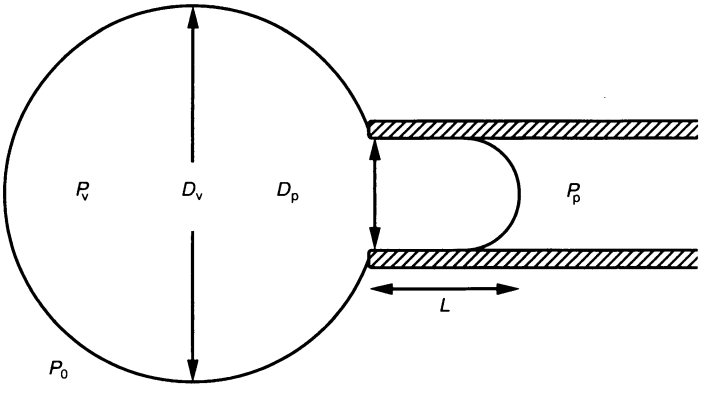


**Figure 1. The pipette aspiration technique**

$P_v$, $P_p$ and $P_o$, pressures in the vesicle, pipette and bathing solution, respectively; $D_p$ and $D_v$, diameters of the pipette and spherical portion of the vesicle, respectively, and $L$, length of the projection into the pipette.

filtration pressures. For erythrocytes pre-swollen in hypotonic solution, as initially used by Evans *et al.* (1976), the pressurization is, in fact, enough for appreciable water loss to occur (Evans & Waugh, 1977). However, with vesicles an order of magnitude larger and comparable membrane tensions, the filtration pressure will be an order of magnitude smaller; and the higher osmolarity of the content of the present vesicles will further help to reduce water loss during aspiration (see also Evans & Needham, 1987). In practice, no change in the length of the projection with maintained constant aspiration pressure was observed.

Equation (1) implies that the cap of the initial projection into the pipette is hemispherical, i.e. that its length is at least equal to the radius of the pipette. For a vesicle to take up this configuration when first lightly aspirated with a pipette about one-third of its diameter, its membrane area needs to be about 2% greater than the minimum required to envelope its volume in perfectly spherical form. Many vesicles satisfied this criterion and a few were even laxer so that they formed initial projections several times the length of the pipette radius. Why vesicles vary in this respect remains unexplored. Unstressed phospholipid vesicles are known to show undulations of thermal origin (Sackmann, Duwe & Engelhardt, 1986), but these probably constitute an excess surface area of less than 1%. In sarcolemmal vesicles, incompletely unfolded membrane invaginations due to residual cytoskeletal elements are more plausible sources of excess membrane. A proportion of vesicles formed, on initial aspiration, only a small bulge into the pipette so that eqn (1) was not applicable. Although it would have been possible to calculate membrane tension and area increase also in such vesicles, they were not used. In later experiments 16 mM sucrose was added to 140 mM KCl in the bath and in the pipette, in order to ensure that all vesicles were adequately deflated. The fact that vesicles were prepared at a higher temperature than that at which they were aspirated would tend to tighten vesicles as the membrane area shrinks more on cooling than does the vesicle content (Kwok & Evans, 1981). But when vesicles were allowed to form at room temperature they were not obviously laxer than those produced by the normal quicker procedure in which the enzyme treatment was conducted at *ca* 34 °C. Laxity was quantified in terms of initial excess membrane area, but no correlation emerged between it and the mechanical parameters of the membrane under stress, and therefore variations in laxity will not be further detailed.

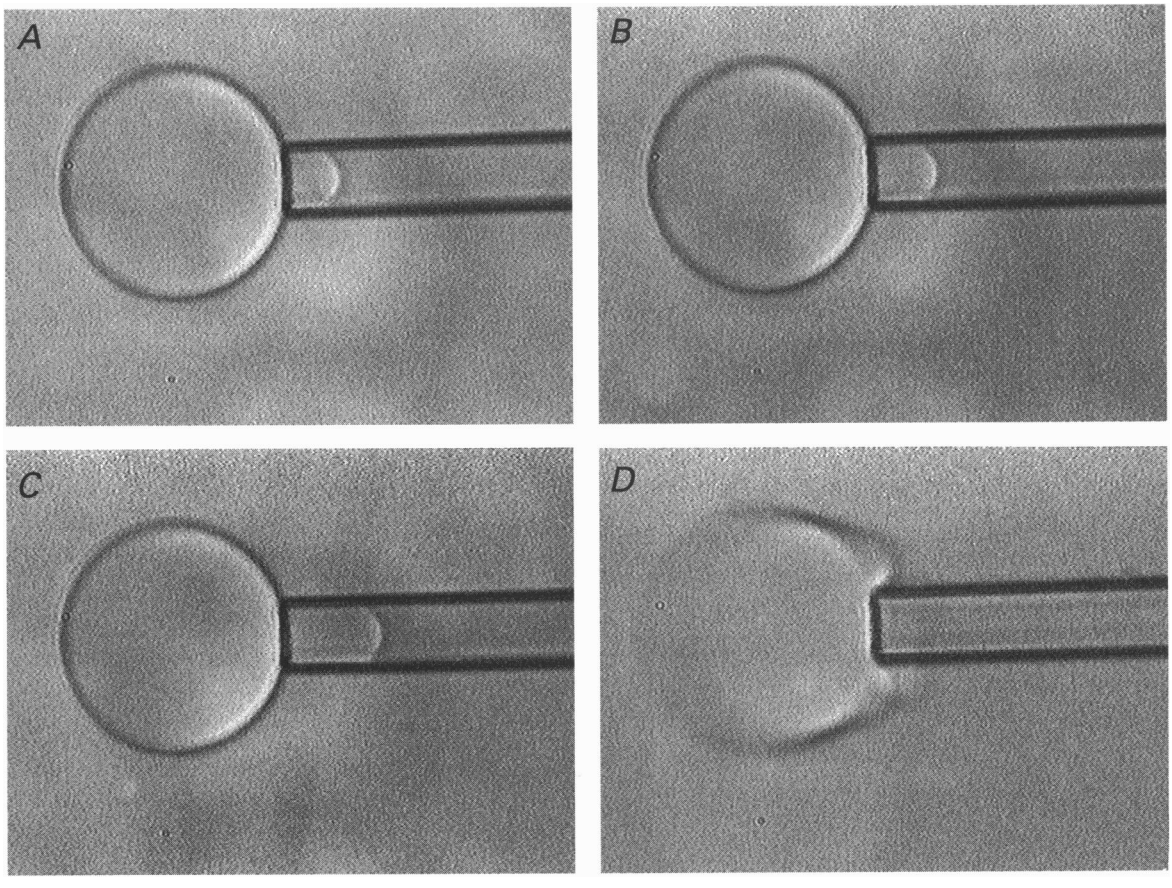


**Figure 2. Video record of a sarcolemmal vesicle during aspiration into a parallel bore pipette**

*A*, a vesicle aspirated with light suction (2 $cmH_2O$) sufficient to take up membrane slack and produce a small projection into the pipette (19 $\mu$m diameter). *B*, suction pressure 8 $cmH_2O$. *C*, suction pressure 20 $cmH_2O$. As the membrane is stressed the projection lengthens upon expansion of the membrane area until a critical point is reached and the vesicle ruptures (*D*). Frames focused for best visibility of projection within pipette. Spherical portion of vesicle (diameter 66 $\mu$m) consequently out of focus.

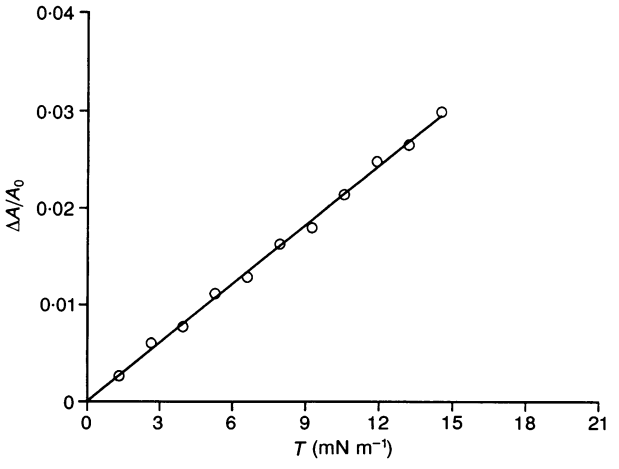


**Figure 3. Stress–strain relationship for a typical vesicle**

Membrane tension ($T$) is plotted against fractional increase in membrane area ($\Delta A/A_0$), for every increment of 2 cm$H_2O$ in suction pressure until vesicle ruptures. Uppermost point denotes lysis tension, $T^*$, and critical area expansion ($\Delta A^*/A_0$).

## RESULTS

Samples of the video record of a typical pipette-aspiration experiment are shown in Fig. 2. The first frame of the sequence shows a vesicle aspirated by a negative pressure of 2 cm$H_2O$, which was generally enough to take up any slack in the membrane. The subsequent frames show how, with increases in aspiration pressure, the projection of the vesicle into the pipette grows longer until sudden rupture occurs.

A plot of membrane tension, $T$, against the fractional increase in membrane area, $\Delta A$, is shown in Fig. 3. Typically, the stress–strain relation is linear right up to the last measurable tension before the vesicle bursts, in this case 14·5 mN m$^{-1}$. This allows extrapolation of the stress–strain relationship to its origin and hence calculation of the unstressed membrane area. In the experiment of Fig. 3 the membrane area had increased by 0·03 of its original unstressed area when the vesicle burst. In subsequent descriptions the maximal sustainable membrane tension (or lysis tension) will be denoted as $T^*$ and the maximum sustainable fractional increase in area as $\Delta A^*$. The point representing $T^*$ and $\Delta A^*$ on $T$ *vs.* $\Delta A$ plots will at times be called the 'lysis point'.

A linear stress–strain relationship signifies that the membrane of sarcolemmal vesicles behaves as a perfectly elastic structure whose distensibility can be defined by the slope of the stress–strain relationship

$$K = T/\Delta A$$

where $K$ is the modulus of area dilatation. In the experiment of Fig. 3 it amounts to 491 mN m$^{-1}$. The area under the stress–strain relation is

$$\frac{T \times \Delta A}{2}$$

and has the dimension of energy. It represents the energy stored per unit area in a vesicle just before it bursts. In the illustrated experiment it amounts to 0·21 (mN m) m$^{-2}$.

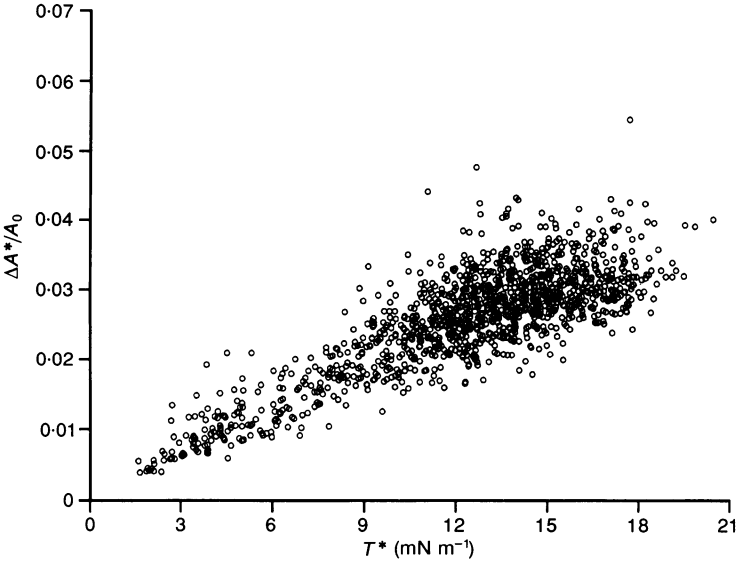


**Figure 4. Distribution of lysis points for control vesicles**

Points show the maximum sustainable tension, $T^*$, and the corresponding fractional increase in membrane area, $A^*/A_0$, for 1301 control vesicles in 140 mM KCl, 5 mM Hepes, at pH 7·4.

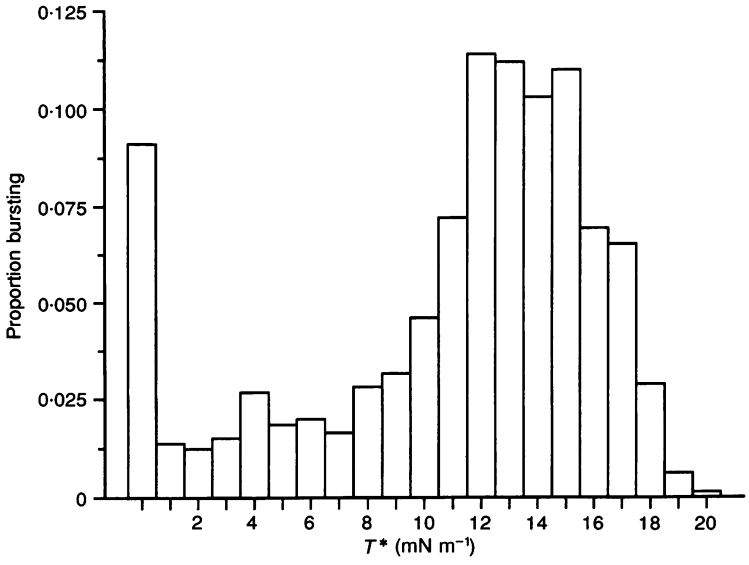


**Figure 5. Probability density function of lysis tension, $T^*$, for control vesicles**
Histogram shows the proportion of vesicles ($n = 1459$) bursting for every increment of 1 mN m$^{-1}$ membrane tension from 0·5 to 20·5 mN m$^{-1}$. The proportion of immeasurably weak vesicles bursting at below 0·5 mN m$^{-1}$ is given by the extreme left-hand bar.

Only on a few early occasions did a day's work consist of measuring the mechanical properties purely of 'normal' vesicles in 140 mM KCl. Usually, a variable was introduced into the experiment. One or two dishes of untreated vesicles were then also prepared and about ten normal vesicles aspirated at intervals during the experiment to serve as controls. In this way, data on over a thousand normal vesicles was accumulated. It is summarized in Fig. 4 in which the lysis point for each normal vesicle is represented.

Figure 4 is incomplete in the sense that it does not include vesicles which were so weak that they burst instantly on aspiration. A few such vesicles were usually present in each batch. A better way to represent the distribution in membrane tensile strength therefore is by means of a probability density function (p.d.f.). This shows that about 8% of vesicles burst at a lysis tension lower than could be readily measured under the experimental conditions normally used (Fig. 5). For the remaining 92% the p.d.f. shows a skewed distribution. An upper limit to the strength of the membrane is obviously imposed by the structure of the membrane. On the other hand, there is no limit to how weak a vesicle can be as a result, say, of faulty sealing during its original formation, or owing to other destructive influences.

A closely related way of representing the distribution of membrane tensile strength is to plot a cumulative distribution function (Fig. 6). This shows the proportion of vesicles bursting at or below a given membrane tension. For the present population of normal vesicles half had burst by 12·4 ± 0·2 mN m$^{-1}$ (median ± 95% confidence limits). The cumulative distribution curve is a compact form of data representation, and in the subsequent paper (Nichol & Hutter, 1996), changes in membrane tensile strength will be represented as shifts in this curve.

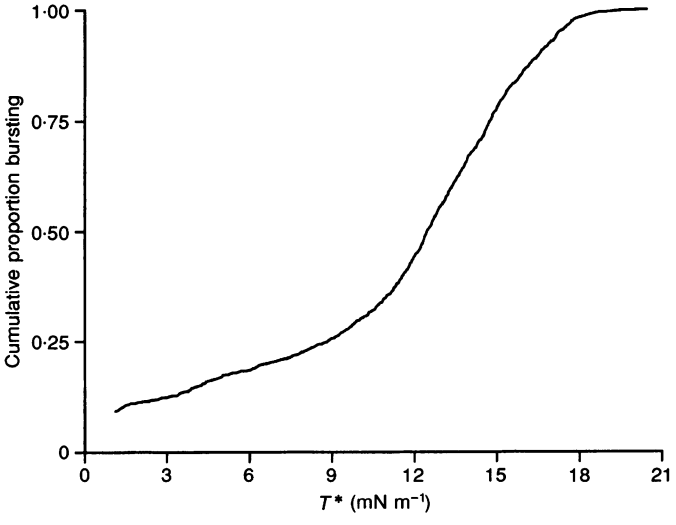


**Figure 6. Cumulative distribution function of lysis tension, $T^*$, for control vesicles**
Graph represents the proportion of vesicles burst at or below a given tension, e.g. half of all vesicles measured burst at tensions below 12·4 mN m$^{-1}$. Proportion of immeasurably weak vesicles is given by the left-hand end of the curve.

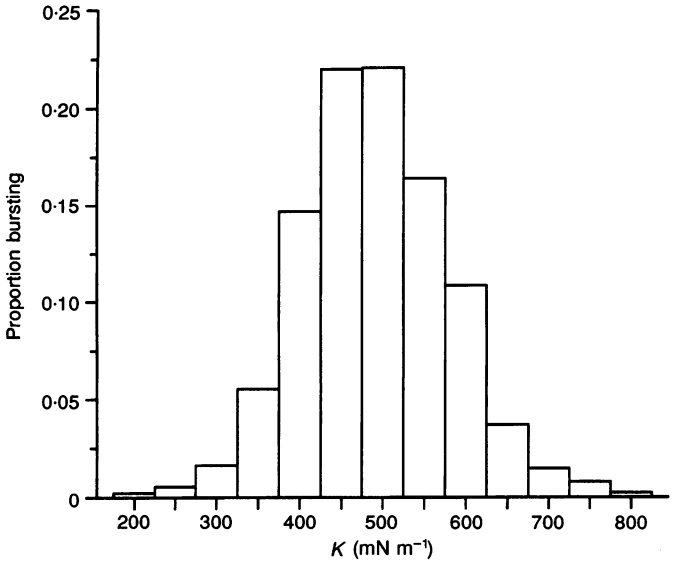


**Figure 7. Probability density function of area elastic modulus, $K$, for control vesicles**

Histogram shows the distribution of $K$ values for 1301 control vesicles about the mean of 490 ± 88 mN m$^{-1}$ and is consistent with a normally distributed function.

An interesting feature of Fig. 4 is the direct relationship between $T^*$ and $\Delta A^*$. Evidently, vesicles greatly differing in the maximum tension which they can sustain are similarly compliant. Nevertheless, the area elasticity of the membrane varies to some extent from vesicle to vesicle even within the same batch. Figure 7 gives the distribution of $K$ for the control population of vesicles already characterized in Fig. 4. Bar a few outriders, $K$ is distributed in approximately normal fashion with a mean value of 490 ± 88 mN m$^{-1}$ (mean ± S.D.)

The distribution of the values of $\Delta A^*$ is shown in Fig. 8. As with the $T^*$ distribution (Fig. 5), it is skewed because vesicles weakened for whatever reason can undergo only a small increase in area before bursting. The median of the distribution was 0·026 ± 0·005 (median ± 95% confidence limits). Only a few of the strongest and most compliant vesicles were able to sustain a fractional increase in membrane area above 0·04.

To test whether the mechanical properties of sarcolemmal vesicles depended on muscle fibre type, vesicles were prepared also from the soleus muscle of a rabbit. The mean values for ten vesicles from that source were $T^* = 13 \pm 1{\cdot}6$ mN m$^{-1}$ and $K = 511 \pm 59$ mN m$^{-1}$, i.e. not distinguishable from the population of normal psoas vesicles.

To test whether the mechanical properties of the sarcolemma are sensitive to the concentration of $Ca^{2+}$ in the bathing solution, 140 mM KCl, to which $CaCl_2$ had been added, was admixed to 140 mM KCl containing shed vesicles, so as to raise $[Ca^{2+}]_o$ to 0·1–1·0 mM. Up to 0·5 mM $Ca_o^{2+}$ no effect was detectable, but vesicles exposed to 1·0 mM $Ca_o^{2+}$ tended to become weaker if measurements were continued for longer than 1 h. The slow time course of this effect suggests that it probably arises from a failure of vesicles subjected to a $Ca^{2+}$ gradient of physiological magnitude to maintain a normally low cytosolic $Ca^{2+}$ level.

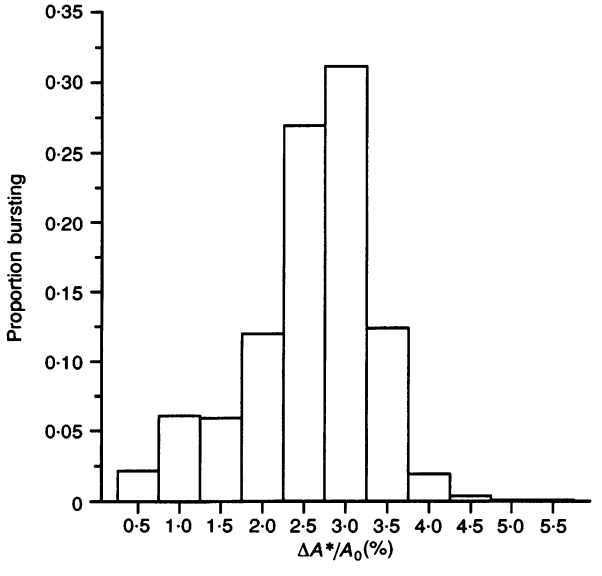


**Figure 8. Probability density function of maximum fractional increase in area expansion, $\Delta A^*/A_0$, for all control vesicles**

Histogram shows the proportion of vesicles bursting at an area expansion of between 0·25 and 5·5 %, in increments of 0·5%. Same population as in Fig. 7.

### Sources of variability

To test how far differences between muscle samples contributed to the total variance of $K$, the results from the 132 experimental days were subjected to one-way analysis of variance. This showed that 31% of the total variance was attributable to day-to-day variations. When the day-by-day results were plotted chronologically it also became evident that $K$ tended to increase over the experimental period (Fig. 9). This trend, when approximated by a linear regression, explained 12% of the total variance. Possibly this trend was due to the overall faster conduct of the experiments which came with experience and which would have minimized any progressive deterioration of vesicles with time. Uncontrolled differences in room temperature from 19 to 25 °C must also have contributed to the day-by-day variance. But as the $Q_{10}$ for $K$ measured between 15 and 30 °C is only 1·2 (E. A. Evans, W. Rawicz & O. F. Hutter, unpublished measurements on murine sarcolemmal vesicles) the variance from that source is unlikely to be more than 4% of the total variance. Only about 15% of the total variance is thus attributable to differences between animals.

With 69% of the total variance in $K$ attributable to scatter within each batch, it may be asked for how much measurement errors can account. This question was addressed by estimating the greatest likely S.D. for each of the measurements entering into $K$ and then synthesizing values distributed normally around an appropriate mean. Values for $K$ were then calculated and their distribution analysed. This procedure takes into account that the errors in the different measurements are not of equal weight, as some measurements enter more than once into the calculation of $K$. Starting with $\Delta P$, this was considered accurate to $\pm 1$ mm$H_2O$. With typical lysis values of around 200 mm$H_2O$, the error from that source was therefore negligible. The error in measuring $D_v$ was also relatively small, say $\pm 2$%. The least accurate measurements entering into $K$ were $D_p$, where an error of $\pm 5$%, i.e. $\pm 1$ $\mu$m for a 20 $\mu$M pipette, was conceivable, and $\Delta L$ where $\pm 5$% again seemed an adequate allowance for error. On this basis, a maximum of 12% of the total variance can be attributed to measurement error. Therefore, more than half the variance in $K$ seems to be due to real but as yet unexplained differences between vesicles, or to unrecognized systematic error.

## DISCUSSION

### Dilatational elasticity

The pipette aspiration technique was first applied to red blood cells transformed into spheres by treatment with hypotonic saline solution (Evans *et al.* 1976). According to Waugh & Evans (1979), the best figure for $K$ based on 200 human red blood cells is 500 mN m$^{-1}$ at 20 °C, i.e. basically the same as found here for rabbit sarcolemmal vesicles. That an intact cell displays membrane mechanical properties so similar to those of sarcolemmal vesicles lends credence to our working hypothesis that sarcolemmal vesicles, though produced by an unphysiological procedure, represent a valid model for studying the muscle surface membrane.

In terms of material science, the area elasticity of the membrane is a continuum property which represents the behaviour of the constituent membrane molecules averaged over space and time (for a review see Bloom, Evans & Mouritsen, 1991). In a composite membrane made up of fluid lipid and relatively rigid integral protein molecules, as is here envisaged for the sarcolemma, the elastic properties will reside in the lipid component (Needham & Nunn, 1990). In the unstressed state the hydrophobic cohesive forces between the hydrocarbon chains will be in equilibrium with the repulsive forces, e.g. those between the head groups of the phospholipid molecules; and the area occupied by each phospholipid molecule will be the optimum area for minimum free energy (Israelachvili, 1985). When stress is applied to the membrane, each phospholipid molecule will come to possess more free energy and occupy a larger area. Israelachvili (1985) has shown that for a phospholipid bilayer expanding elastically the modulus of area elasticity $K$ (mN m$^{-1}$ or mJ m$^{-2}$) will be four times the minimum free energy due to hydrophobicity of each monolayer, which latter can be estimated to be about 50 mJ m$^{-2}$. For a phospholipid bilayer a value for $K$ of about 200 mN m$^{-1}$ would therefore be expected. Values of $K$ in the order of 500 mN m$^{-1}$, as found in red

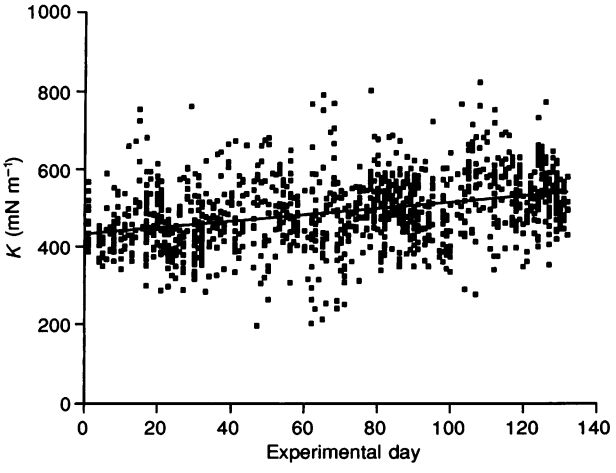


**Figure 9. Linear regression analysis of $K$ data for control vesicles**

Values ($n$ = 1301) of area elastic modulus, $K$, are plotted, in chronological sequence, against the experimental day ($n$ = 132) on which they were measured. Positive correlation between $K$ and experiment number is shown by the least-squares regression line $y = 0{\cdot}824x + 433{\cdot}4$.

blood cells and as here also reported, must therefore be attributed to the presence in the sarcolemma of (i) cholesterol which intercalates as a smooth, hard face between phospholipid molecules and so straightens acyl chains and improves cohesion (Bloom *et al.* 1991) and (ii) of integral membrane proteins which are largely incompressible and so give rise to a higher value of $K$ than would be obtained if the membrane were composed of lipids alone.

Of particular interest in the above connection are the measurements of Evans & Needham (1987) and Needham & Nunn (1990) on the mechanical properties of vesicles made of lipid extracted from red blood cells, which contains about 40 mole % cholesterol. For such vesicles they found $K$ to be 470 mN $m^{-1}$, that is essentially the same value as for normal red blood cells (Waugh & Evans, 1979). This was surprising because an appreciable fraction of the membrane surface is occupied by rigid proteins, and hence the lipid matrix must be more distensible than the composite membrane. Needham & Nunn (1990) reconcile these facts by supposing that the proteins of the membrane confer enhanced distensibility upon the red blood cell lipid by perturbing the lipid bilayer in the anular region.

The surface membrane of cells in general is known to be rich in cholesterol (Jain, 1988), and it seems reasonable to assume that integral membrane proteins will occupy a similar proportion of the total membrane area in sarcolemmal vesicles as they do in the red blood cells. By analogy, we may therefore conclude that the mechanical properties of sarcolemmal vesicles are determined by (i) the composition of the lipid matrix of the membrane and (ii) the degree to which the presence of rigid membrane proteins on the one hand reduces the distensibility of the composite membrane and, on the other hand, perturbs the lipid matrix of the membrane. In the interpretation of changes in the mechanical properties of sarcolemmal vesicles, such as will be described in the subsequent paper (Nichol & Hutter, 1996), consideration will therefore be given to possible changes in the above factors.

### Lysis tension and critical areal strain

A consistent finding in the present work has been that untreated vesicles of similar distensibility may burst at greatly different stresses and hence area expansion. A likely explanation is that membrane rupture results from the development of a weakened spot and that this process is dominated by local fluctuations in membrane composition. Two mechanisms can be invoked: (i) local thinning of membrane lipids and (ii) lateral movement of membrane proteins floating in the fluid membrane into a configuation which reduces membrane strength. In view of the interaction between membrane lipids and proteins, these two processes are not necessarily distinct. Rather, lipid packing defects at lipid–protein interfaces may result from protein aggregation. At all events, membrane failure under tension probably results from stochastic processes (Evans *et al.* 1976), so there would be a finite possibility of catastrophy occurring early during pipette aspiration. With progressive increase in the applied stress, a progressively smaller and hence more frequently occurring packing defect would then suffice to cause membrane rupture. If lysis tension were determined purely probabilistically, i.e. if the population of vesicles were fully homogeneous, the p.d.f. for lysis tension should be perfectly bell-shaped, and the cumulative distribution function perfectly sigmoid. But in fact these functions were skewed with an excess of weak vesicles (Figs 5 and 6). It must therefore be supposed that a proportion of vesicles are flawed for additional reasons, possibly related to their initial formation.

As regards critical areal strain, sarcolemmal vesicles also resemble red blood cells: for both, $\Delta A^*$ is about 0·03. The explanation offered for this low tolerance is that lipid molecules cease to cohere as soon as water molecules can enter between them. Finally, it might be added that the above finding is in no way inconsistent with the well-known ability of isolated muscle fibres to lengthen under passive stretch by up to nine times the resting length without membrane injury (Dulhunty & Franzini-Armstrong, 1975). The latter ability is explained by the fact that in an intact muscle fibre the membrane is folded and caveolated and this provides the spare membrane for fibre elongation.

### Does $[Ca^{2+}]_o$ influence membrane tensile strength?

Sarcolemmal vesicles contain ATP (Burton *et al.* 1988) and are probably capable of glycolysis, but it is as yet unknown how well they can defend themselves against imposed ionic gradients. For the mechanical measurements reported here, vesicles were therefore normally left in the solution in which they formed, i.e. in nominally $Ca^{2+}$-free KCl. This also ensured comparability with the data on membrane mechanical properties of red blood cells which were similarly measured in saline without added $Ca^{2+}$ (Evans *et al.* 1976; Waugh & Evans, 1979). However, it was clearly necessary to test whether membrane mechanical properties are altered by lack of extracellular $Ca^{2+}$. That addition of up to 500 $\mu$M $Ca_o^{2+}$ had no effect accords with the view that membrane tensile strength is determined by the state of the lipid matrix; and that the zwitterionic phospholipids interspersed with cholesterol, which mostly comprise the external leaflet of cell surface membranes, have little tendency to bind $Ca^{2+}$ (Shah & Schulman, 1967). On present evidence, membrane strength is thus distinct from membrane ionic permeability, where some proteinaceous membrane channels require $[Ca^{2+}]_o$ to retain their normal properties (Almers, McCleskey & Palade, 1984; Armstrong & Miller, 1990). As to the small reduction in membrane

tensile strength observed on prolonged exposure to 1 mM $Ca_o^{2+}$, our interpretation that this probably arises from accumulation of $Ca^{2+}$ within vesicles is borne out by the results of the accompanying paper (Nichol & Hutter, 1996).


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