Cytoskeletal dynamics and spontaneous inside-out vesiculation of red blood cell membranes

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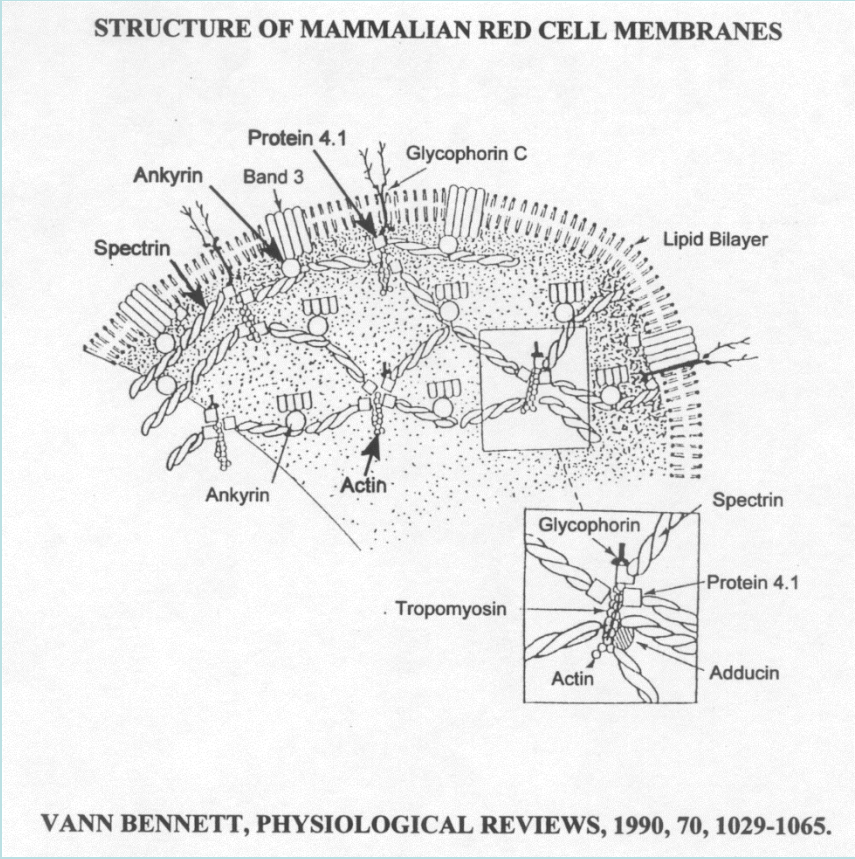
Cytoskeletal dynamics during spontaneous inside-out vesiculation

1. Background on IOV formation
2. Video clips showing spontaneous inside-out vesiculation
3. An investigation of the role of cytoskeletal dynamics in IOV formation

Cortical cytoskeleton responsible for...

- ***Known and familiar roles:***
- Physiological shape control and normal dynamic folding in the microcirculation
- Pathological shape alterations: echynocytosis, sickling, etc
- ***Exotic dynamics:***
  - **Malaria-induced (*mechanisms still unexplained*):**
    - **Merozoite contacts induce large and dynamic RBC deformations as part of the apical alignment process before invasion (Video clip 2, Glushakova et al.)**
    - **merozoite egress: violent rupture, curling, buckling, eversion and vesiculation in less than 0.5s (Video clips 3 and 4, Abkarian et al.)**
  - **Spontaneous inside-out vesiculation (*incipient understanding of the mechanism*)**

A bit of background...



- Ted Steck and his team in the early seventies developed a method to generate IOVs which became a widely used tool in studies of transport enzymes in RBC membranes (Steck & Kant, Methods in Enzymology, 1974. 31:172-180).
- They showed that the main protein components of the cortical cytoskeleton, spectrin and actin, were absent from the IOVs

Background continued...

- SK-IOVs were prepared by lysing RBCs in divalent cation-free DW with very low concentrations of buffer to pH 7.5-8.0, followed by lengthy washes and cold-incubation steps lasting about two days

- ***In the eighties we discovered that IOVs form spontaneously within minutes of RBC lysis enabling us to follow and record IOV formation under the microscope in real time***

- ***We investigated how these spontaneously formed IOVs were generated (***Lew et al., Nature. 1982. 296:742-744; Lew et al., Nature. 1985. 315, 586 589; Lew et al., 1988, Journal of Cell Biology, 106, 1893-1901; Tiffert et al., J. Physiol., 487.P, 99P; Tiffert and Lew, Pflugers Arch - Eur J Physiol. 2014. DOI)

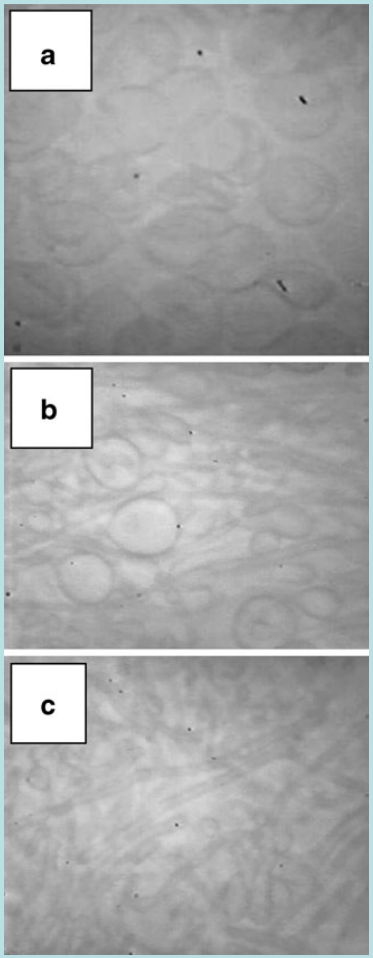
- ***We proposed a molecular mechanism in which the disassembly of the spectrin-actin cytoskeleton played critical roles in IOV formation***

Trying to make sense of how IOVs were formed

- **There were two hypotheses at the time:**
- ***By extensive endocytosis*** (Steck)
- ***By shearing of a giant liposome***: Because IOVs lacked the main cytoskeletal proteins, spectrin and actin, Branton and Shotton suggested that vesiculation conditions cause the cytoskeleton to disintegrate first leaving a ***giant liposome***. By shearing this giant liposome through thin needles the membrane ruptured into myriad vesicles, mostly of IO topology
- However, these hypotheses were incompatible with what we were seeing, as you will appreciate presently
- But let's start by looking at what happens to membrane dynamics in ghosts with spectrin-actin removed, as with the hypothesized ***giant liposomes***

Video-clip 1: Behaviour of spectrin-actin-free RBC membranes under flow

(Method of Tiffert and Lew, Eur J Physiol. 2014, DOI 10.1007/s00424-014-1483-5)



So, without cytoskeleton...

- The membrane has no intrinsic shape
- And no intrinsic dynamics
- The membranes deform along thin tubular shapes to minimize flow frictional resistance under convective currents
- Their behaviour corresponds to that of a two-dimensional liquid structure
- So, in principle, the Branton-Shotton hypothesis of IOV formation by giant liposome shearing appeared feasible

May be feasible, but is it real?
Let’s see...

Spontaneous vesiculation of RBC membranes

RBCs are lysed in large volumes of divalent cation free DW pH 7.5 at 0^{o}C, the pelleted membranes are placed between slide and coverslip and observed under phase contrast (x 1000) on a stage at 37^{o}C (video clip 2)

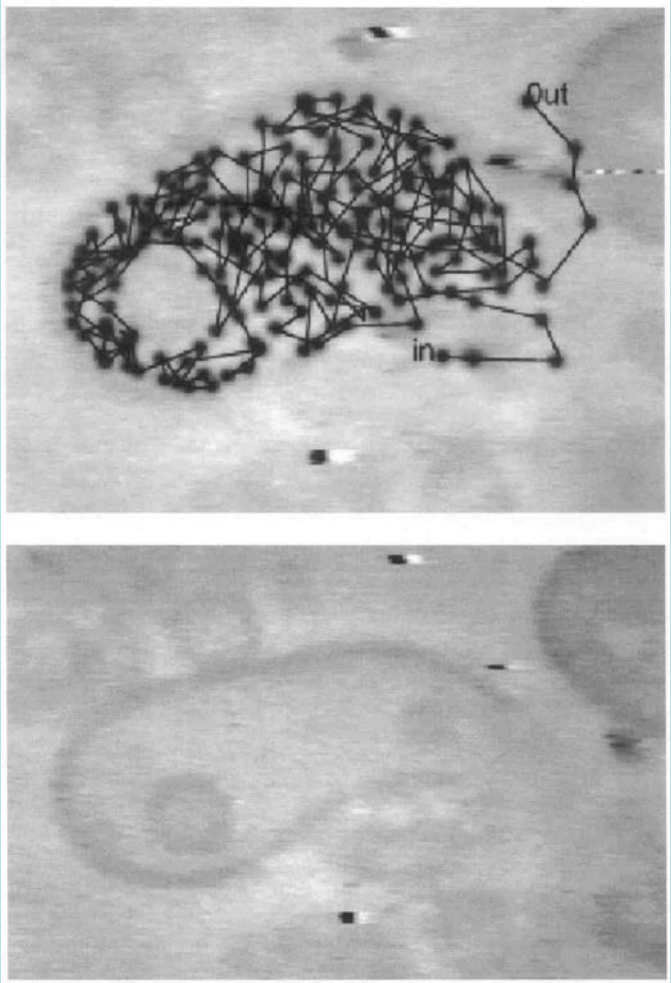
Same field 1m 40s later...
(video clip 3)

Membrane curling outwards at open edge
(video clip 4)

Are the openings real? Mg^{2+} reversibly blocks vesiculation allowing visualization of stabilized intermediate stages. Here we use 0.4 µm diameter latex particles in Brownian motion to explore whether openings are real (video clip 5)

Are the large membrane openings formed at the instant of lysis? (video clip 6)

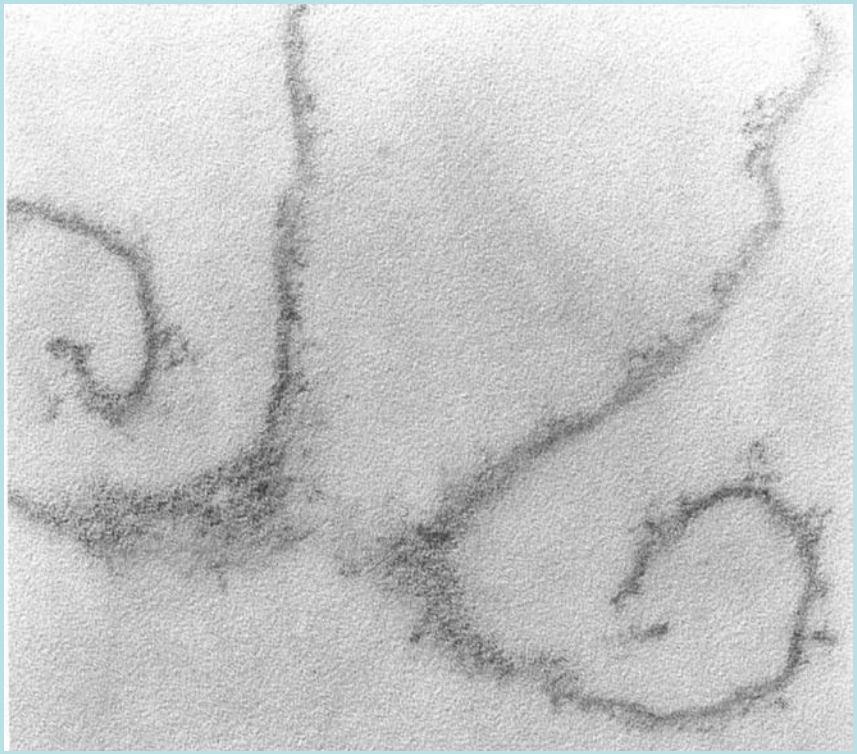
The openings are real

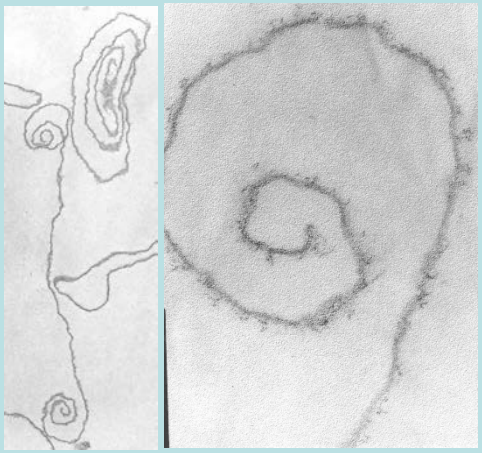


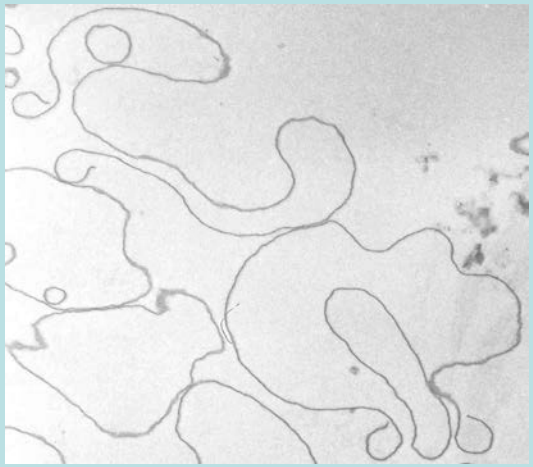
- The opening are large
- They are present in the ghosts in the pre-vesiculation state
- They are present throughout the intermediate stages of vesiculation

Serial section EM studies of membranes in initial, intermediate and final vesiculation stages allowed us to develop a molecular model of IOV formation

(Lew et al., 1988, Journal of Cell Biology, 106, 1893-1901)







The early studies and video record showed that...

(Lew et al., 1988, Journal of Cell Biology, 106, 1893-1901)

- ***Vesiculation was not by endocytosis***
- ***Vesiculation was not through giant liposome formation***
- ***Vesiculation was a spontaneous phenomenon***
- ***Each ghost was converted into a "bunch of grapes and tubules" within 5-8 min at 37^{o}C***
- ***Ghosts opened on lysis with a variable fraction everting fully***
- ***The membrane curls out, with IO topology***
- ***Vesiculation occurs mostly near these openings***
- ***The toroid around the open end is the main IOV factory***
- ***The energy source for the membrane motions must reside within the membrane because the only other energy source in the system is thermal***
- ***Is cytoskeletal disassembly driving vesiculation?***

If cytoskeleton disassembly...

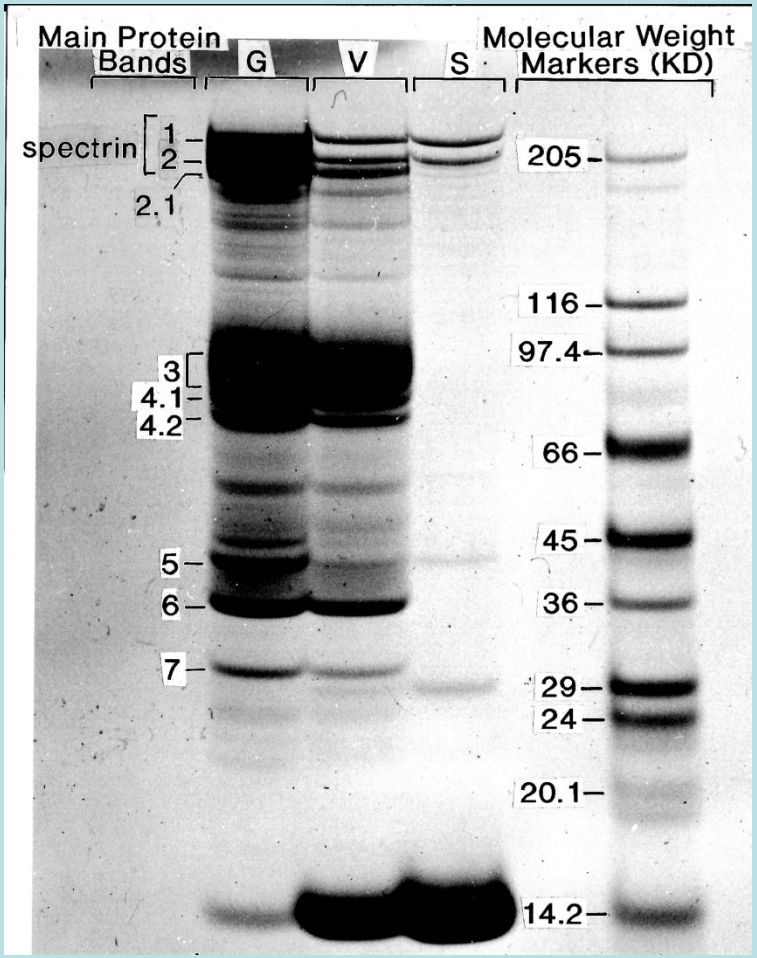
- ***drives the spontaneous membrane motions***
- ***shapes vesicular formation***
- ***sustains the membrane tensions required for keeping large open membrane configurations***

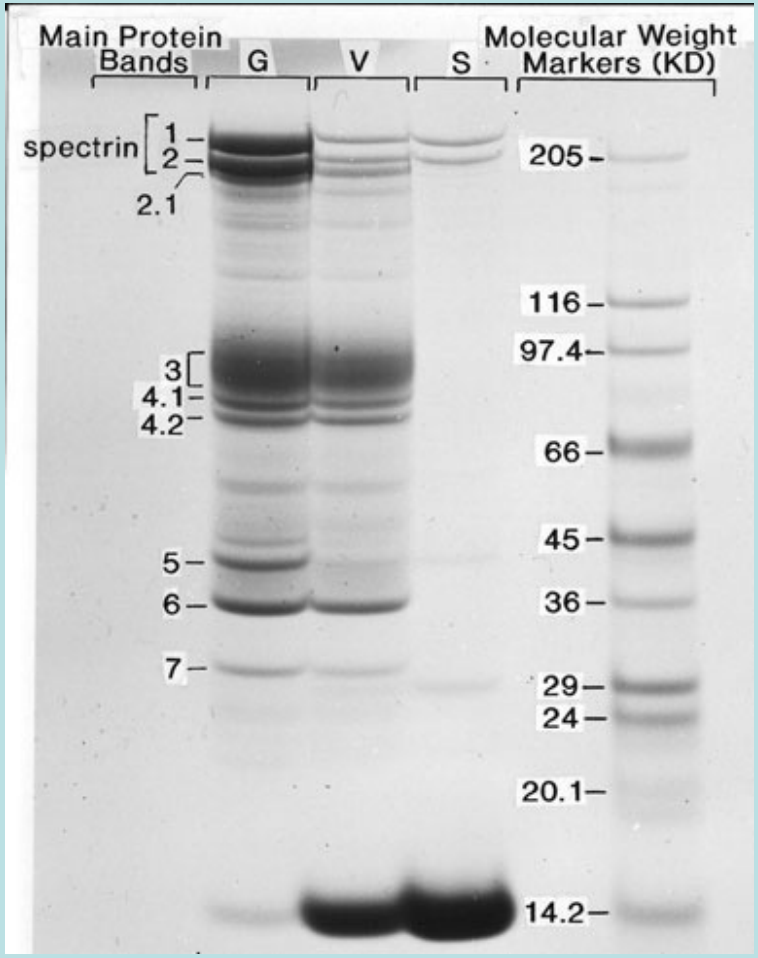
Then spectrin and actin must remain attached to the membranes while spontaneous motions last

To investigate this...

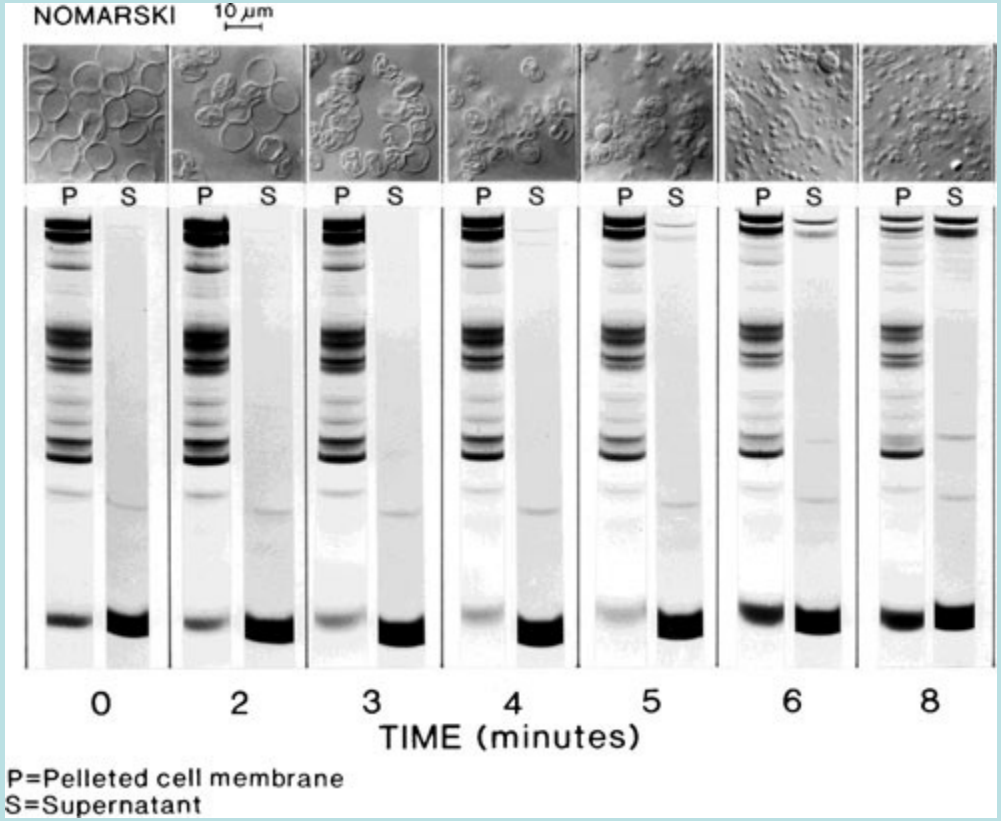
- *Follow time-course of membrane protein changes in **synchronized** ghost suspension, using Mg^{2+} to block vesiculation at intermediate stages*
- *Use marker to indicate the instant of terminal vesicular formation and sealing (haemoglobin trapping)*

Membrane proteins before and after spontaneous inside-out vesiculation:
SDS–gel electrophoretic patterns of pre-vesiculation ghosts (G), post-vesiculation IOVs (V) and post-vesiculation supernatants (S)

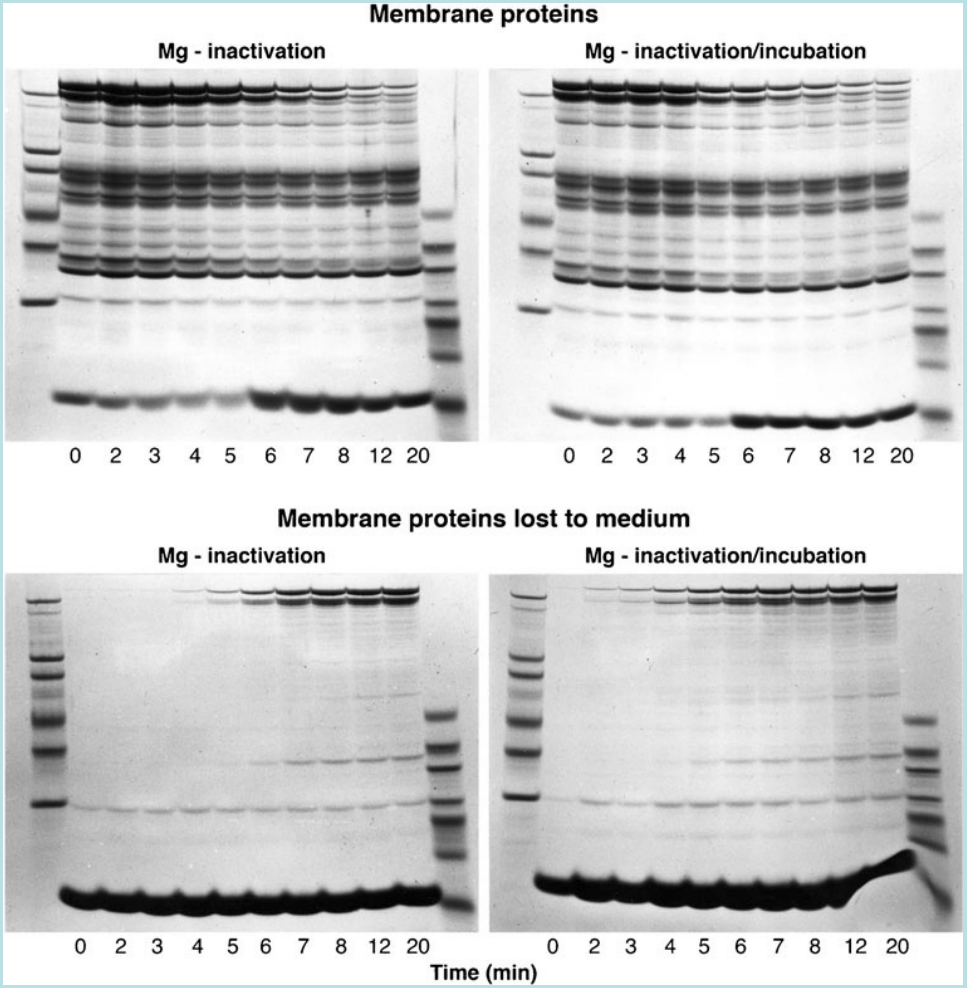




Time relations between morphological and membrane protein changes during spontaneous inside-out vesiculation of red cell membranes



Effect of magnesium ions on the time-dependent patterns of membrane protein changes during spontaneous vesiculation



- Notwithstanding imperfect synchronization, it is clear that there is no large-scale spectrin loss from membranes to supernatants during the first 4 to 5 min, the most dynamic stage of the spontaneous vesiculation process.
- Large-scale spectrin loss occurs concurrently with haemoglobin retention in the membrane protein gels (6-min sample) reflecting vesicular sealing.

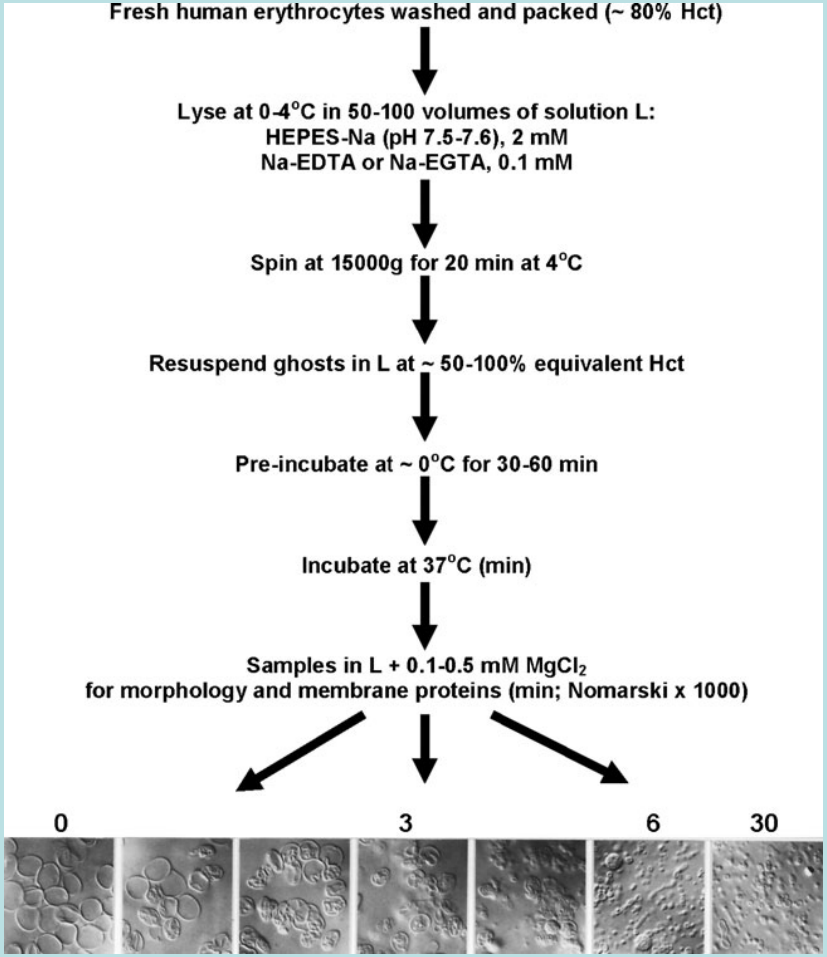
Conclusions

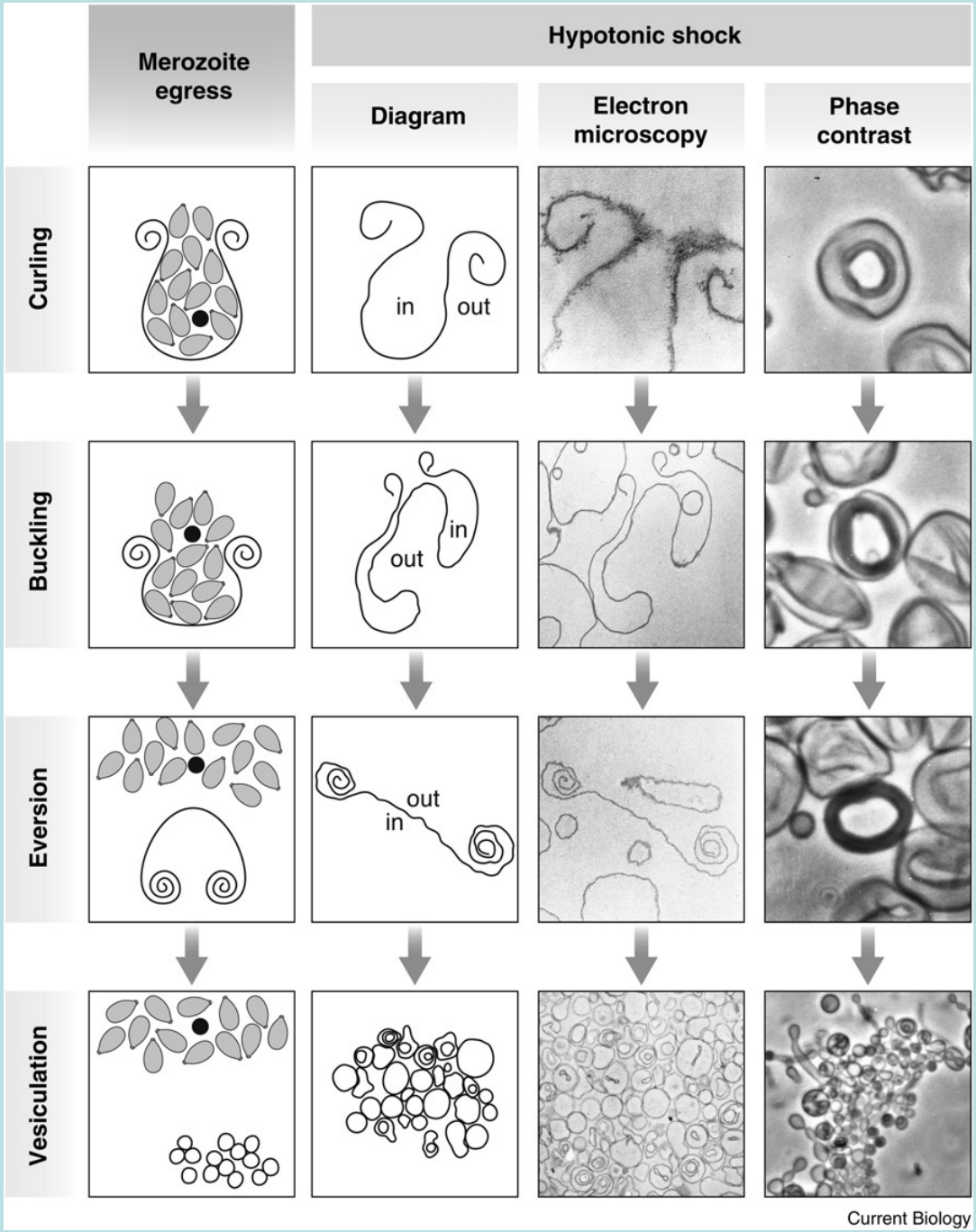
- ***Despite imperfect synchronization it is clear that spectrin-actin are retained while membrane motions persist, as expected if cytoskeletal disassembly is the "muscle" driving and shaping IOV formation***

- ***Spectrin-actin detachment is coincident with vesicular sealing***

- ***This supports the role of the cytoskeleton in the maintenance of the membrane tensions that are needed to sustain open membrane configurations prior to terminal vesicle sealing***

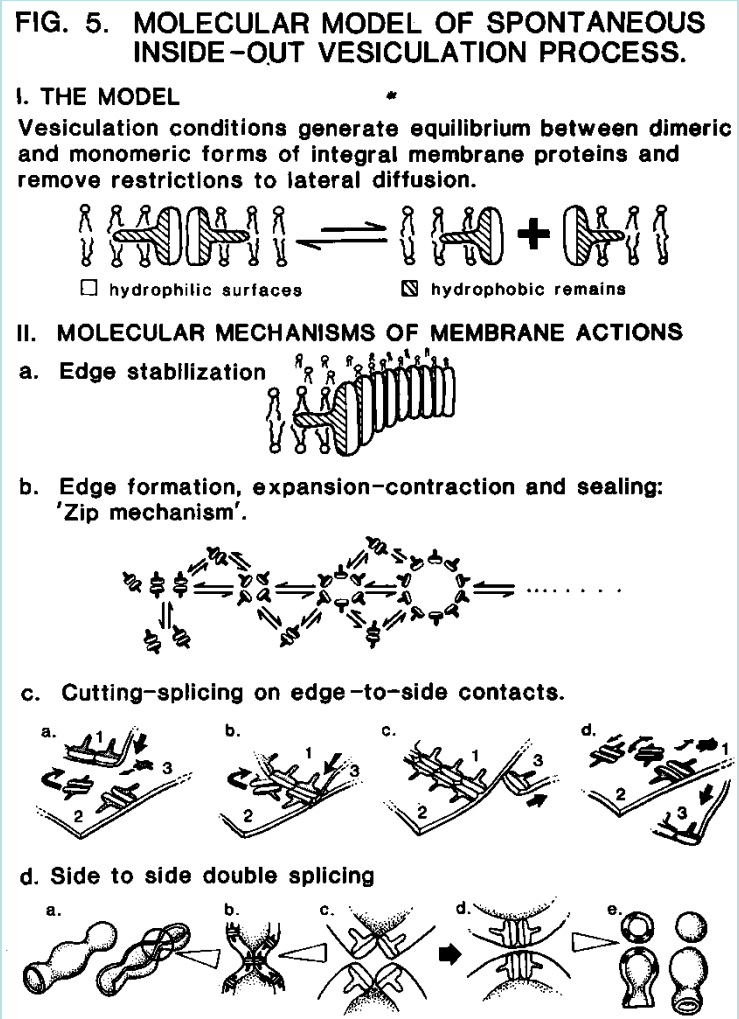
Spontaneous vesiculation of red blood cell membranes: Protocol




Merozoite egress
Hypotonic shock
Diagram
Electron microscopy
Phase contrast
Curling
Buckling
Eversion
Vesiculation
in
out
out
in
out
in
Current Biology

Following a detailed investigation of the mechanism of IOV formation we formulated a working hypothesis of how free edges and vesicles were generated

(Lew et al., 1988, Journal of Cell Biology, 106, 1893-1901)



Merozoite-induced deformations during apical alignment

Glushakova et al., Current Biology, 2010, 20, 1–5

- Video clip Glushakova

Egress of falciparum merozoites

Abkarian et al., Blood. 2011;117(15):4118-4124

- Video clip Abk1

And looking at the RBC membrane alone, labelled with a fluorescence marker (PKH26)

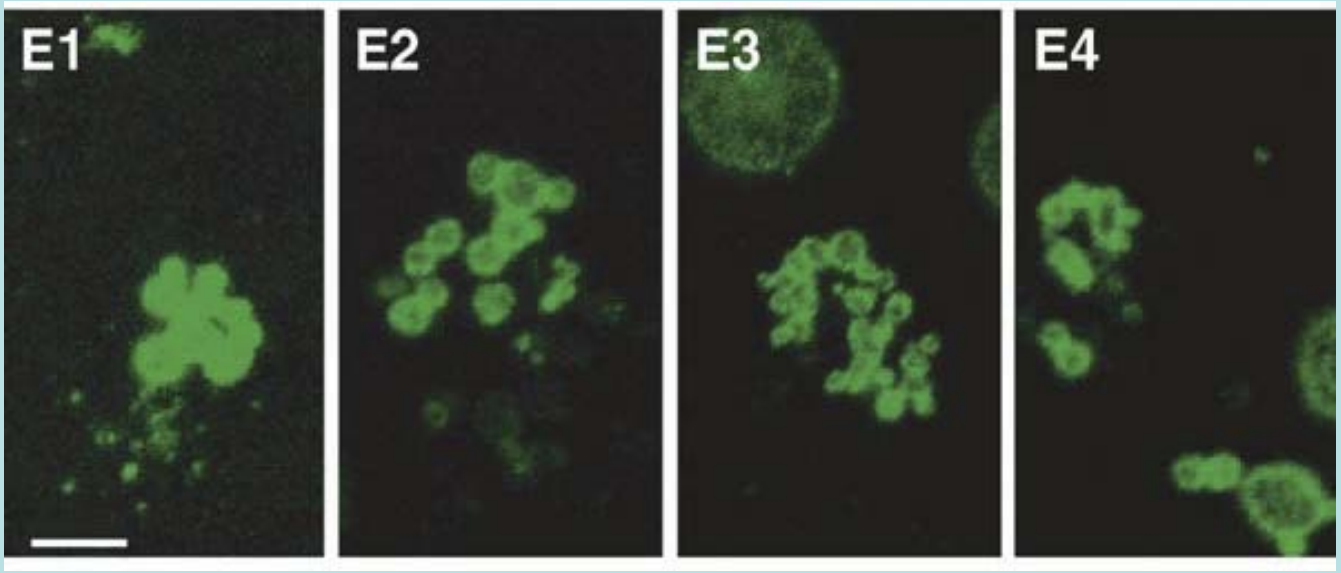
Abkarian et al., Blood. 2011;117(15):4118-4124

- Video clip Abk2

The parasite somehow conditions the cytoskeleton so that the merozoites can be released with minimal hindrance to their dispersal, by making the RBC membrane follow a dynamic sequence of

- ✓*Rupture*
- ✓*Outward curling of the open edges into a toroid shape*
- ✓*Rapid backward eversion and buckling*
- ✓*Final vesiculation of the residual membrane*

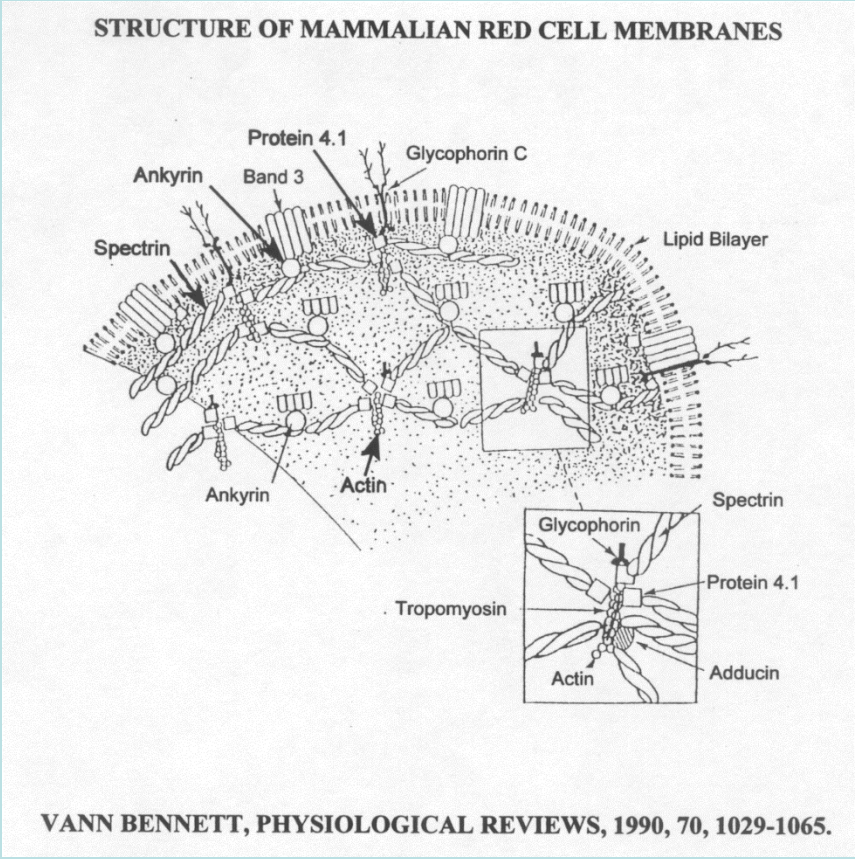
Vesiculated condition of the residual RBC membrane after merozoite egress

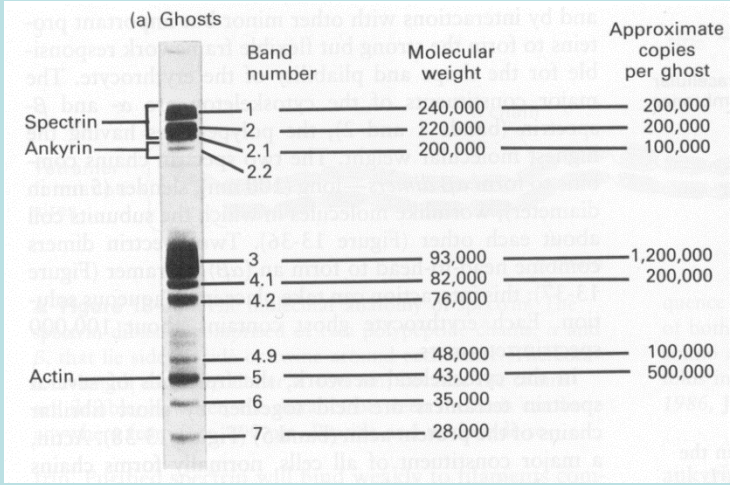


- From Glushakova et al., 2005, Current Biology, Vol. 15, 1645–1650

- Biotinylated surface of RBC is labelled with streptavidin-QD525 (green)
- Different patterns of RBC vesiculation at the sites of release.
- Note vesicles of different sizes and different states of aggregation.
- Some fragments could be composed of membrane blebs.

The two-dimensional cytoskeleton of human RBCs





Are these membrane configurations unique to RBC membranes?

Proc. Nat. Acad. Sci. USA
Vol. 72, No. 10, pp. 3952–3955, October 1975
Biochemistry

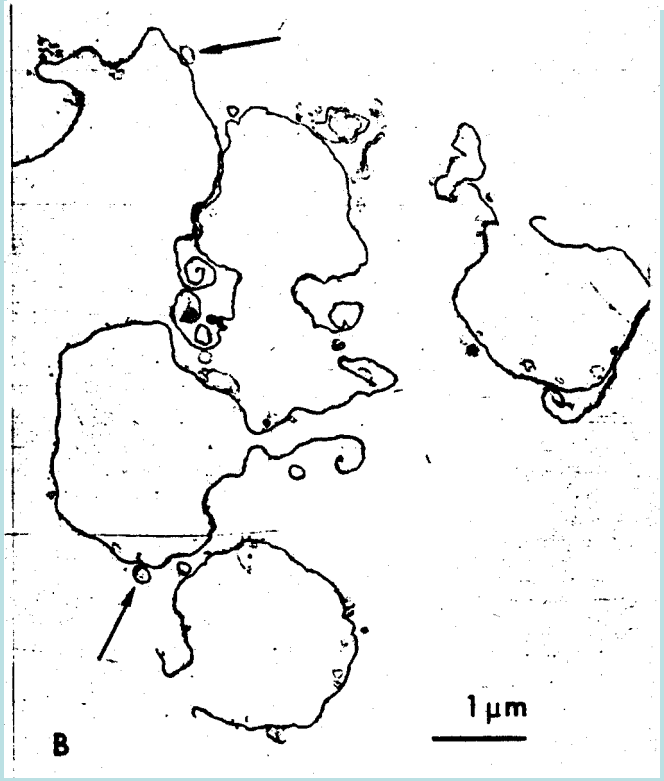
Chitin synthetase zymogen is attached to the yeast plasma membrane

(yeast septum/gradient centrifugation/concanavalin A/glutaraldehyde)

ANGEL DURÁN*, BLAIR BOWERS†, AND ENRICO CABIB*‡

* National Institute of Arthritis, Metabolism and Digestive Diseases, and † National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

Communicated by T. M. Sonneborn, August 4, 1975



- Fig 2B of this paper shows open plasma membrane fragments from yeast isolated in conditions substantially different from those of vesiculating RBC membranes

Can other proteins stabilize free edges?

Fig. 1. Sectioned pellet containing exclusively small membrane fragments which appear flat or cupshaped.

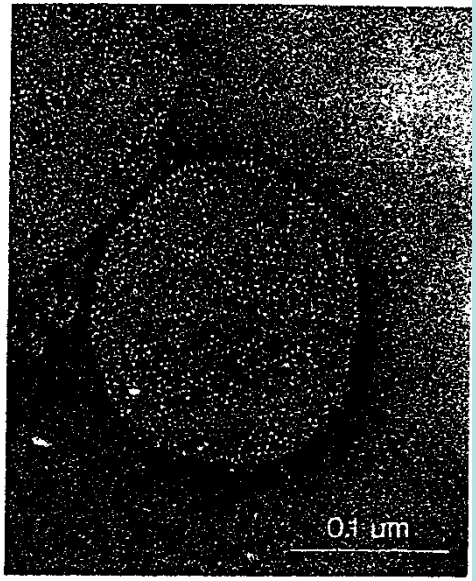
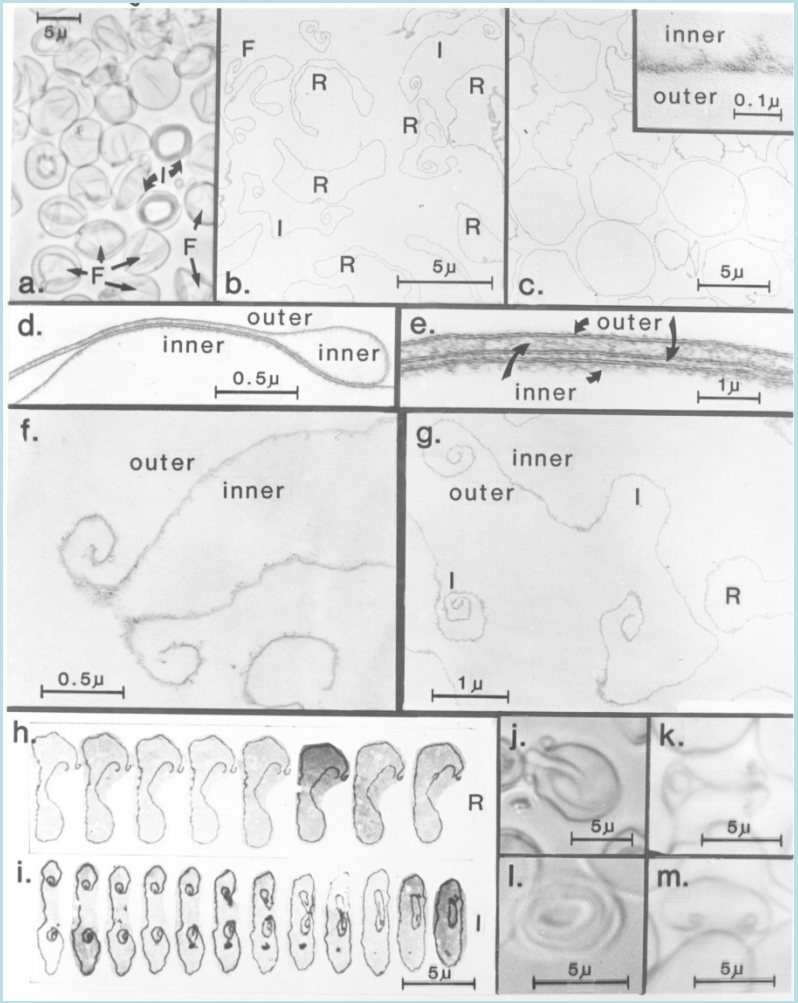


Fig. 2. Negative staining of membrane fragments showing particles arranged in clusters and strands.

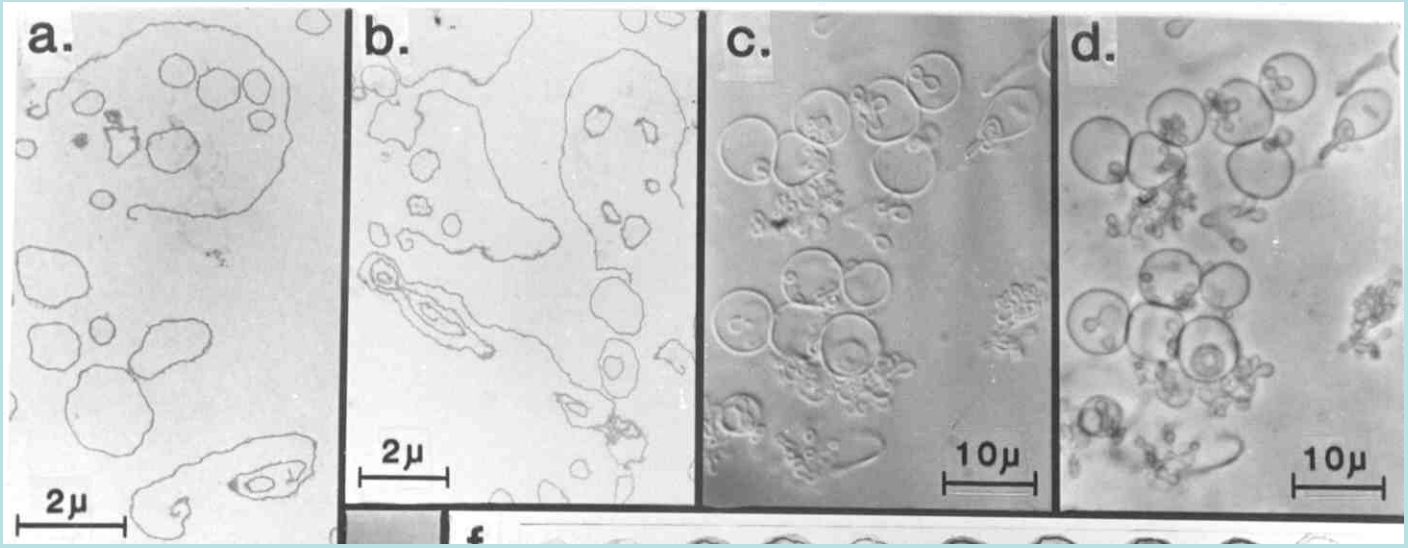
- Deguchi, N., Maunsbach, A.B. and Jorgensen, P.L. 1974. Ultrastructure of Pure ($Na^+ + K^+$)-ATPase membranes. Biochem. Biophys. Acta. 356, 36.

The initial, pre-vesiculation stage



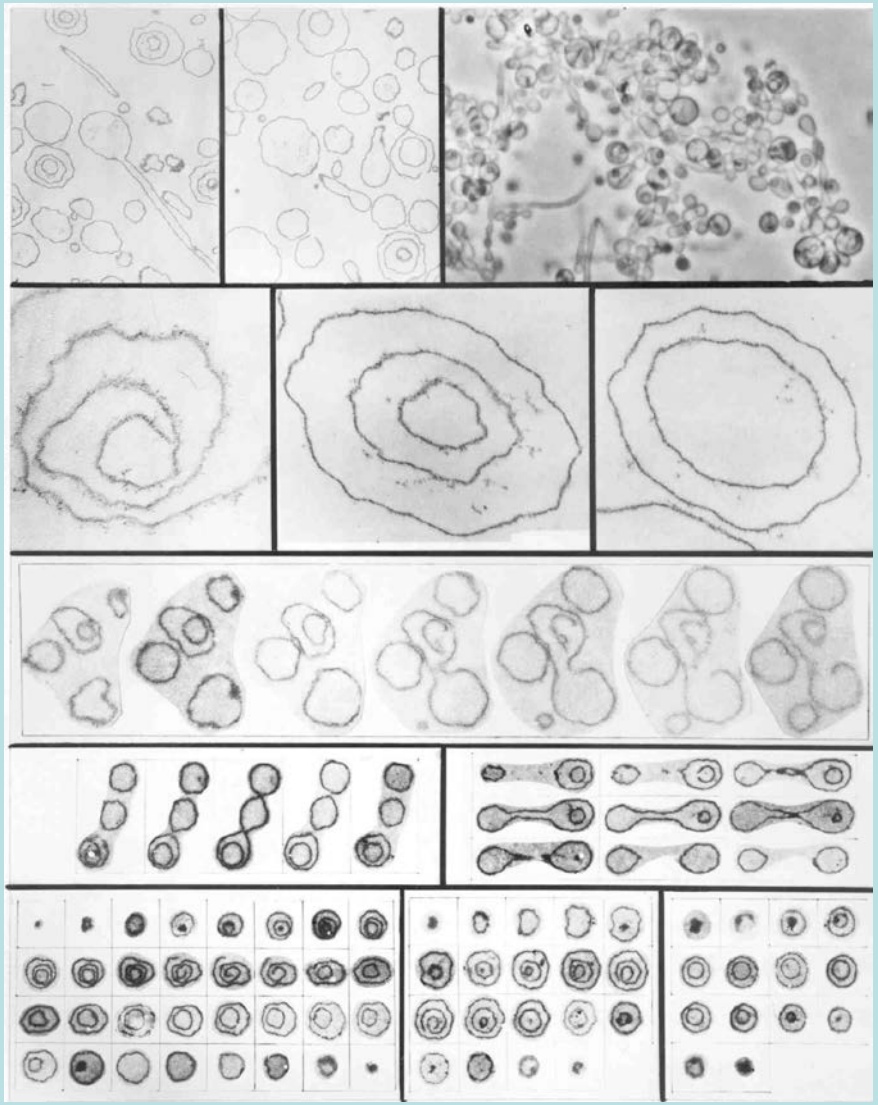
- Fibrillar projections define membrane sidedness
- IOGs and ROGs
- Serial sections containing over 70% of ghost area always show openings
- EM images of fixed specimens are compatible with optical images of fresh samples

Intermediate stages



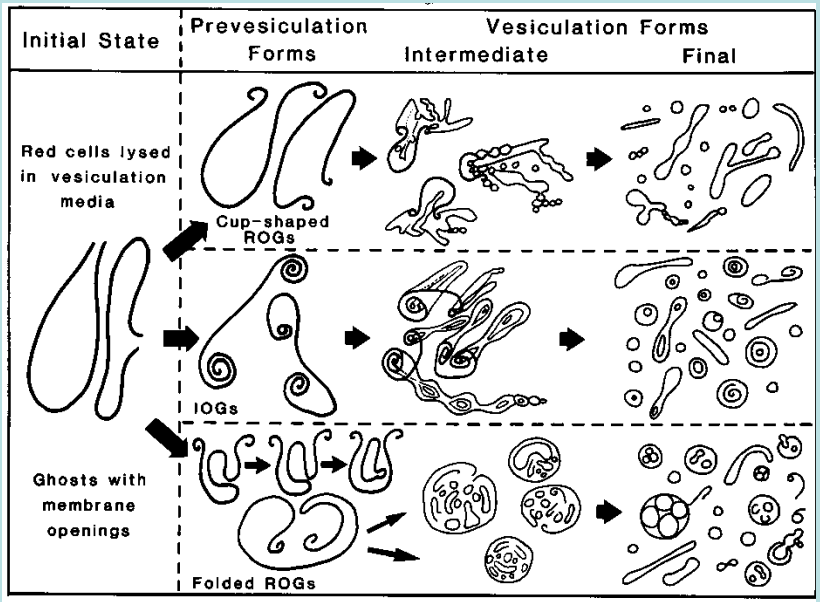
- Note that the reduced membrane fragments preserve the original ROG and IOG configuration during vesiculation
- …as if vesiculation occurred mainly at the IO-curled edges

The final stage



- Note the bewildering variety of vesicle shapes
- Note the reduction or disappearance of the fibrillar projections in the final vesicles

The vesiculation process

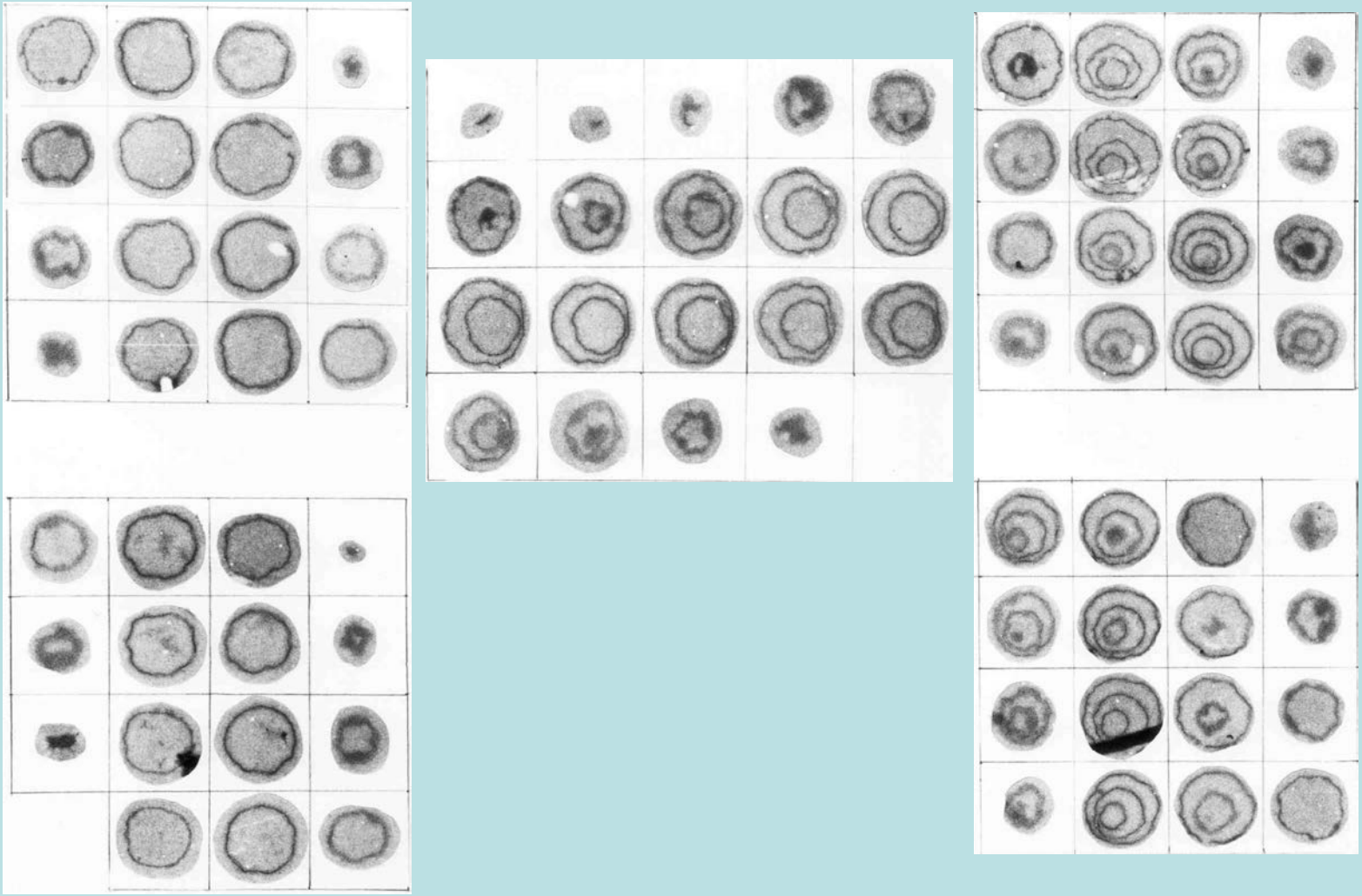


B

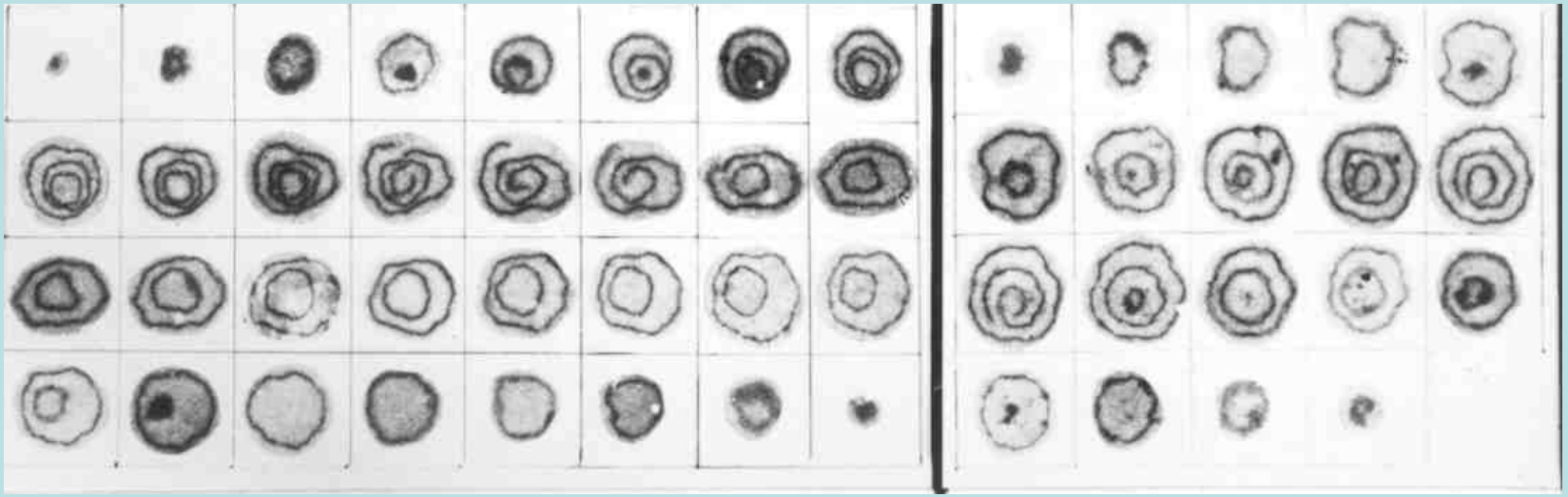
	Stages of Vesiculation Process	Membrane Actions	Diagrammatic Representation of Membrane Actions
I	Formation of concentric spheroids from spiral segments	Cutting-splicing on edge to side contact	a. b. c. d. e. f.
II	Detachment of closed cylinders	Cutting-splicing on edge to side contact	
	Detachment of open cylinders	Edge formation in regions of opposing forces	
III	Fragmentation of cylinders and vesicles	Cutting-splicing on side-to-side contacts	Fragmentation
	Fusion of folds		Fusion
IV	Closing of membrane gaps	Edge reduction	

- Three pre-vesiculation open-ghost configurations give rise to the bewildering variety of vesicle shapes observed
- Free membrane edge formation and cutting-splicing processes can account for the dynamic geometry of spontaneous vesiculation in all its for
- The new challenge: *what plausible molecular model could explain this extraordinary geometry?*

Substantial fractions of vesicles are closed

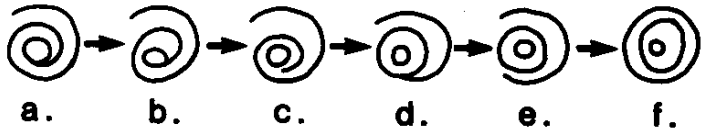


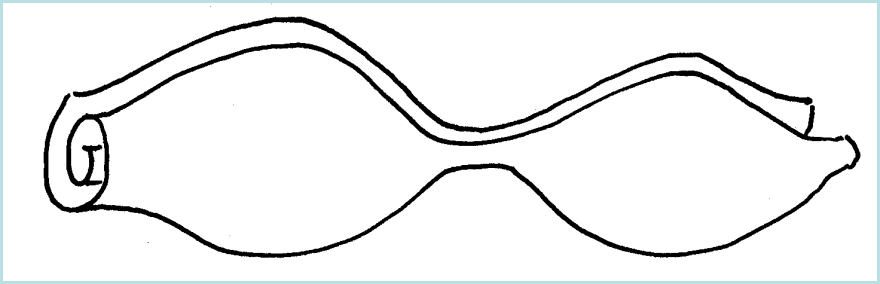
But some are not…



- What process generates the difference between closed and open vesicles?
- Let’s apply the tailor’s approach to this particular case using scissors and tape

Cutting-splicing on edge to side membrane contacts

I	Formation of concentric spheroids from spiral segments	Cutting–splicing on edge to side contact	



- Sealed vesicles: when cutting splicing progresses from one source-point only
- Open spirals: two or more non-matching point-sources

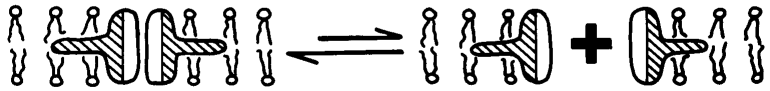
The “tailor” approach

- Assume the membrane is a cloth of constant area and try to reconstruct the vesiculation process using scissors and tape following the geometrical transformations documented in the serial section studies
- Try to figure out a molecular model that can reproduce the tailor’s job

FIG. 5. MOLECULAR MODEL OF SPONTANEOUS INSIDE-OUT VESICULATION PROCESS.

I. THE MODEL

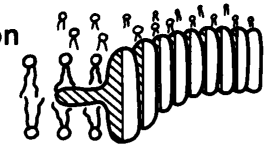
Vesiculation conditions generate equilibrium between dimeric and monomeric forms of integral membrane proteins and remove restrictions to lateral diffusion.



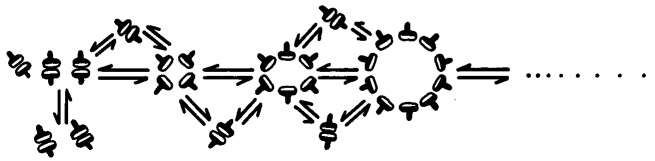
☐ hydrophilic surfaces ▨ hydrophobic remains

II. MOLECULAR MECHANISMS OF MEMBRANE ACTIONS

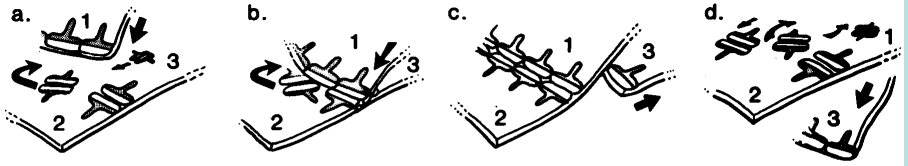
a. Edge stabilization



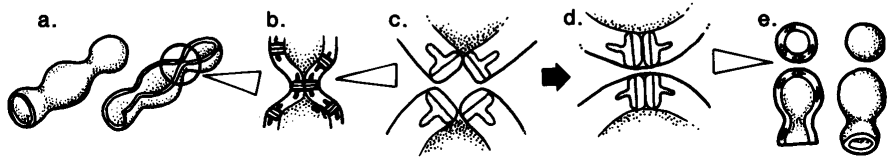
b. Edge formation, expansion-contraction and sealing: 'Zip mechanism'.



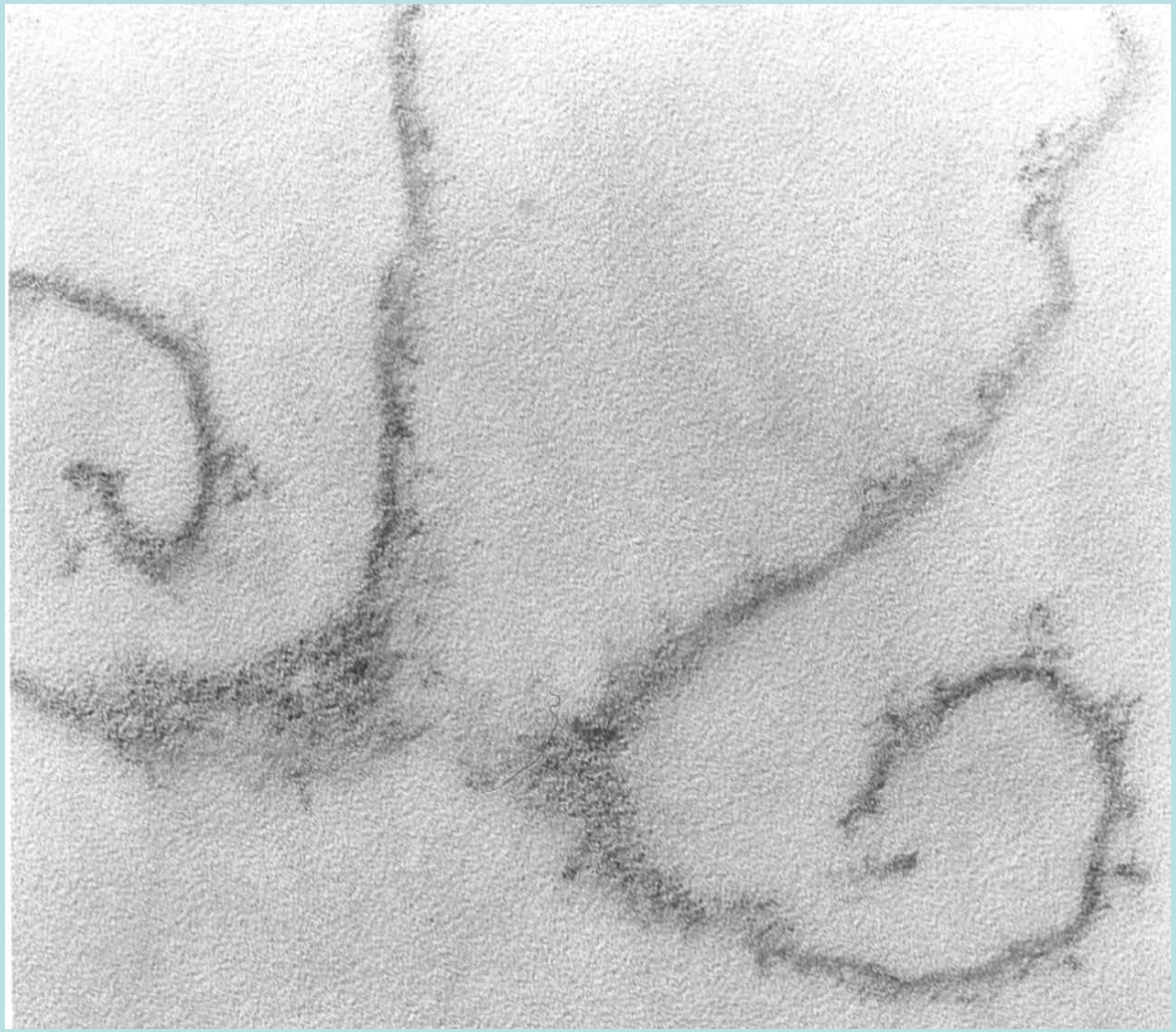
c. Cutting-splicing on edge-to-side contacts.



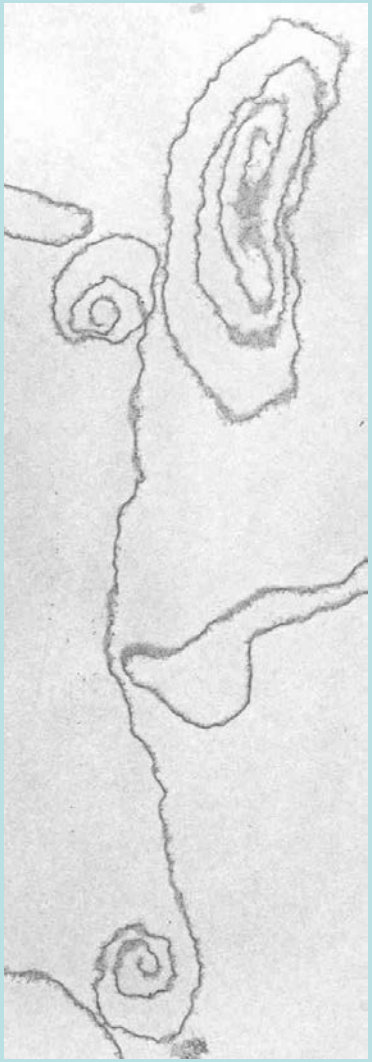
d. Side to side double splicing

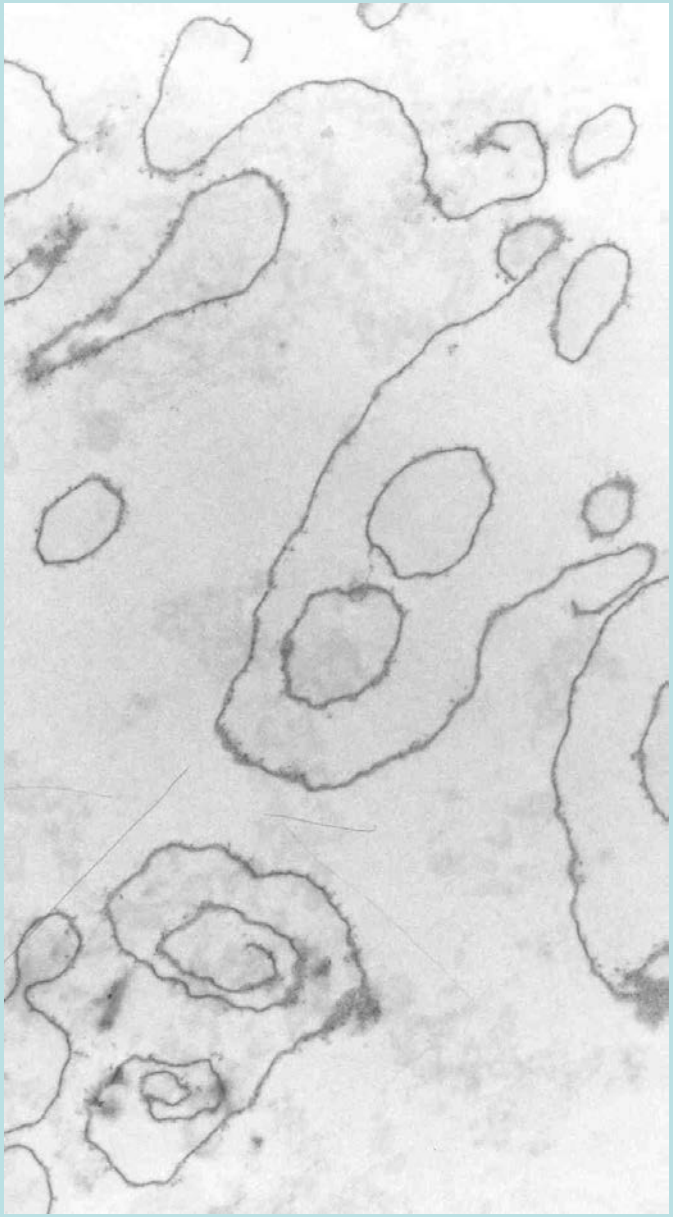


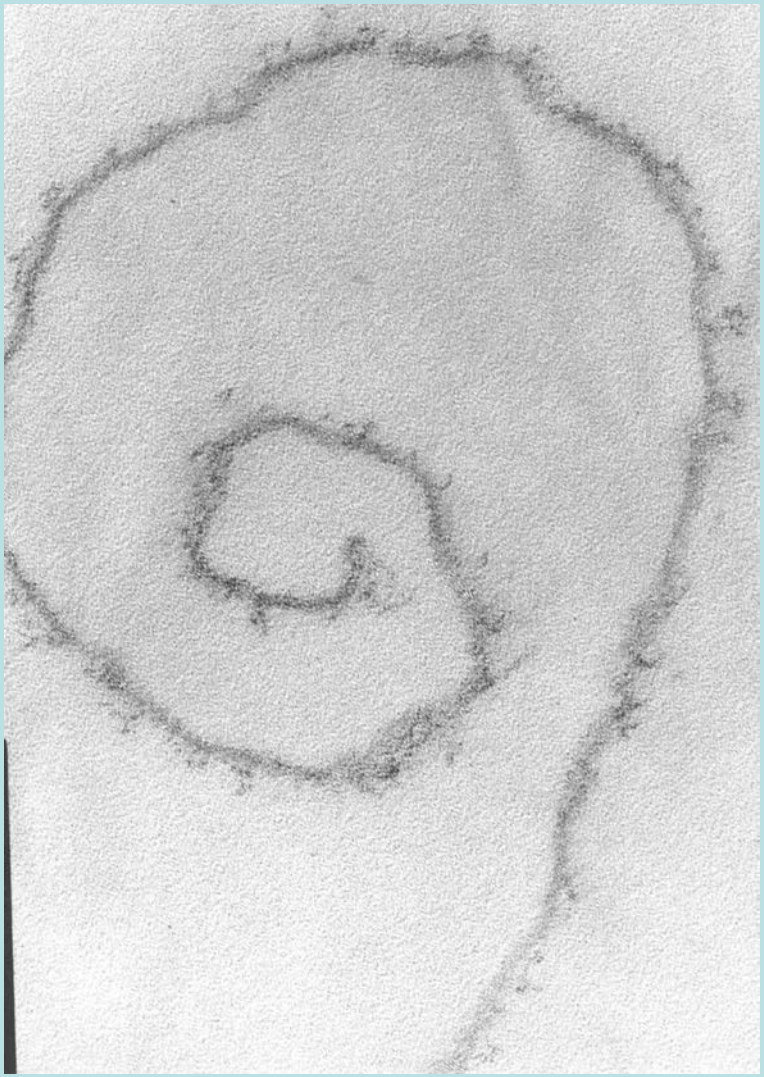
The open end of ROGs



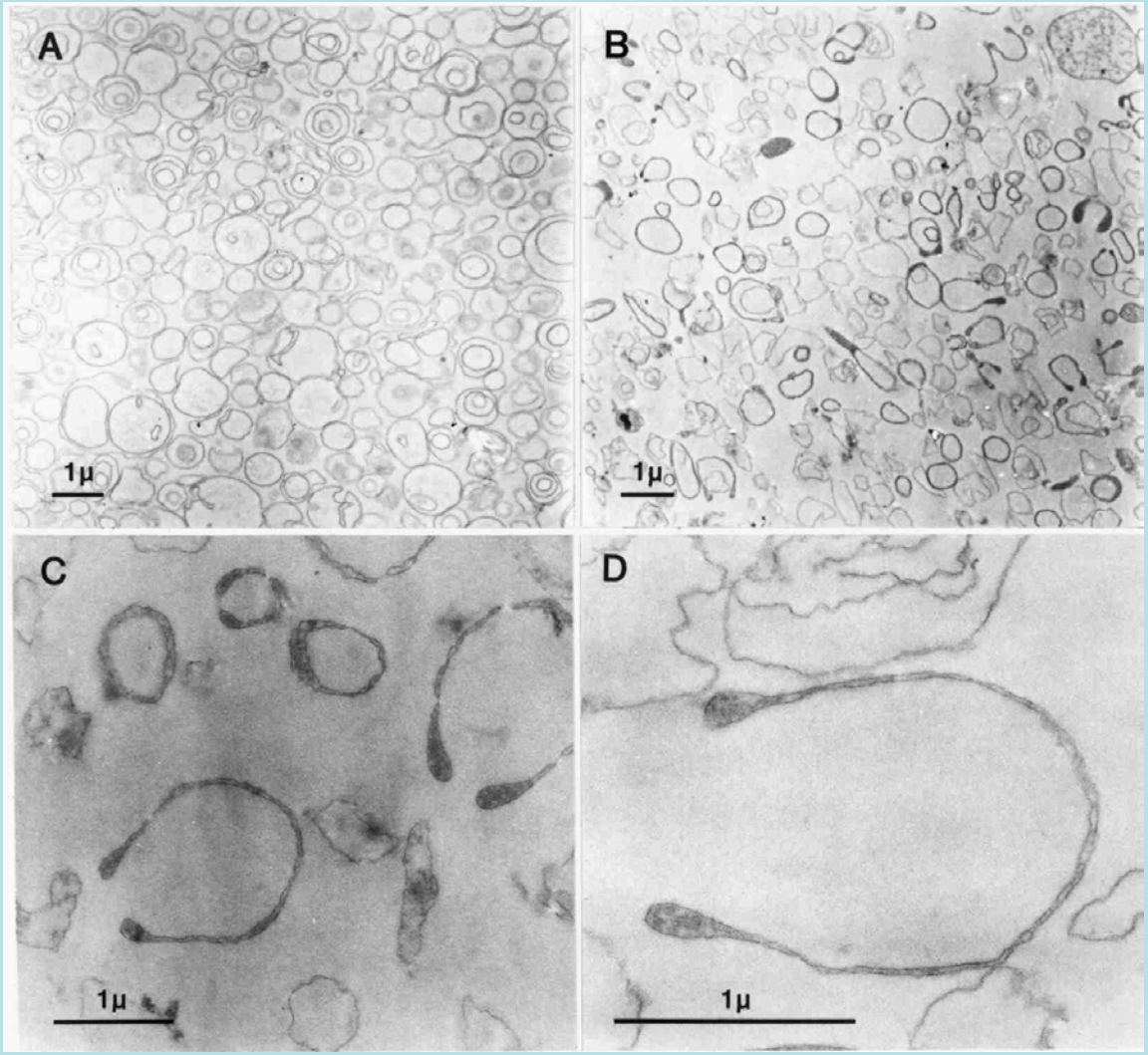
Everting ghosts

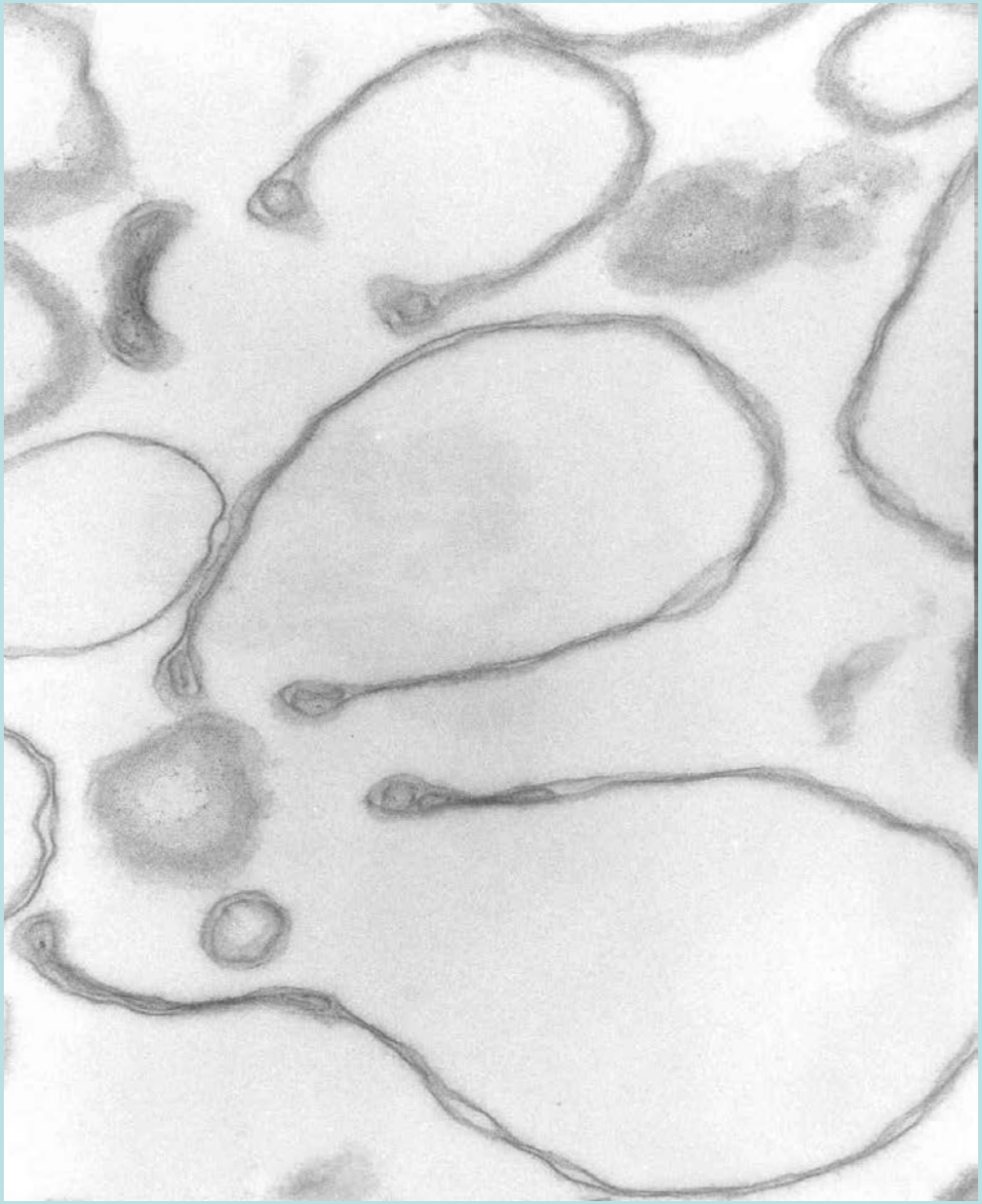






Osmotic vesicle collapse





Open questions, twenty years on…

- The monomer-dimer hypothesis of free-edge stabilization and cutting-splicing is yet to be tested

- One-step IOV-formation does not denature or functionally alter any of the enzymes and transporters of the native red blood cell tested so far

- Can some of the processes involved in spontaneous vesiculation occur in physiological conditions?

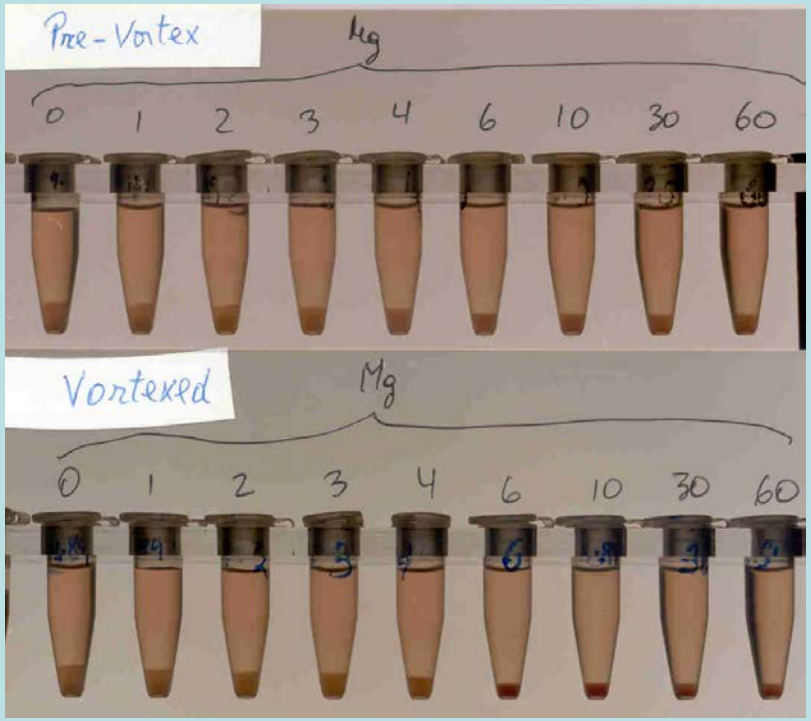
At constant membrane area the volume of vesiculating membranes ought to decrease ~ linearly with mean vesicle radius

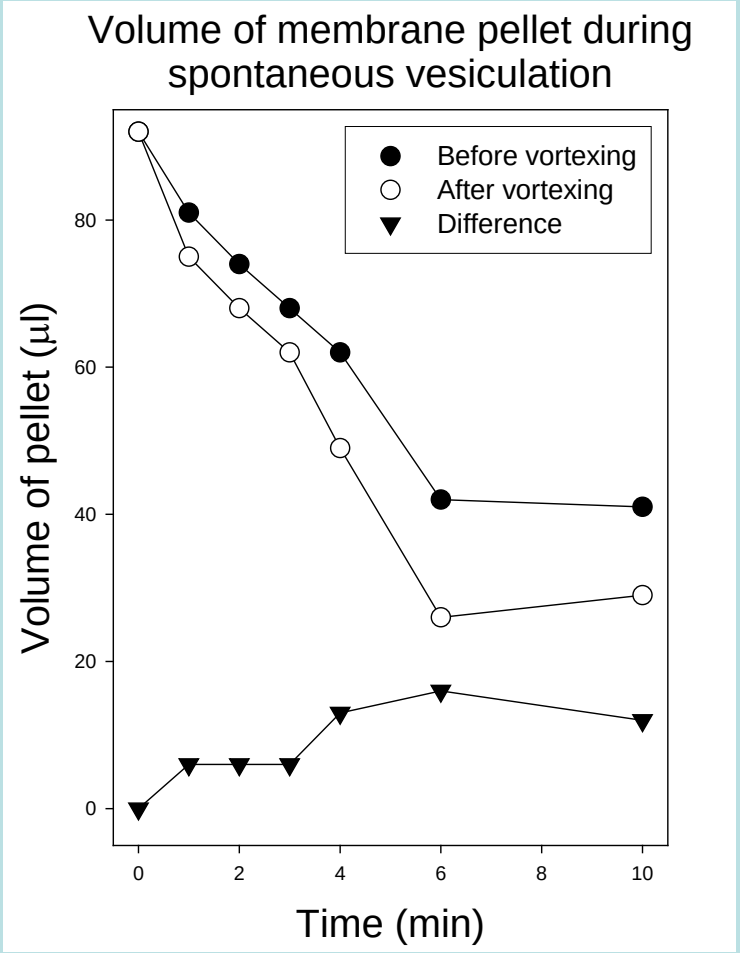
$$V = \frac{4}{3}\pi r^3$$

$$A = 4\pi r^2$$

$$\frac{V}{A} = \frac{r}{3}$$

Estimate of the fraction of cytoskeleton-free membrane area available for further vesiculation by shearing forces during spontaneous vesiculation

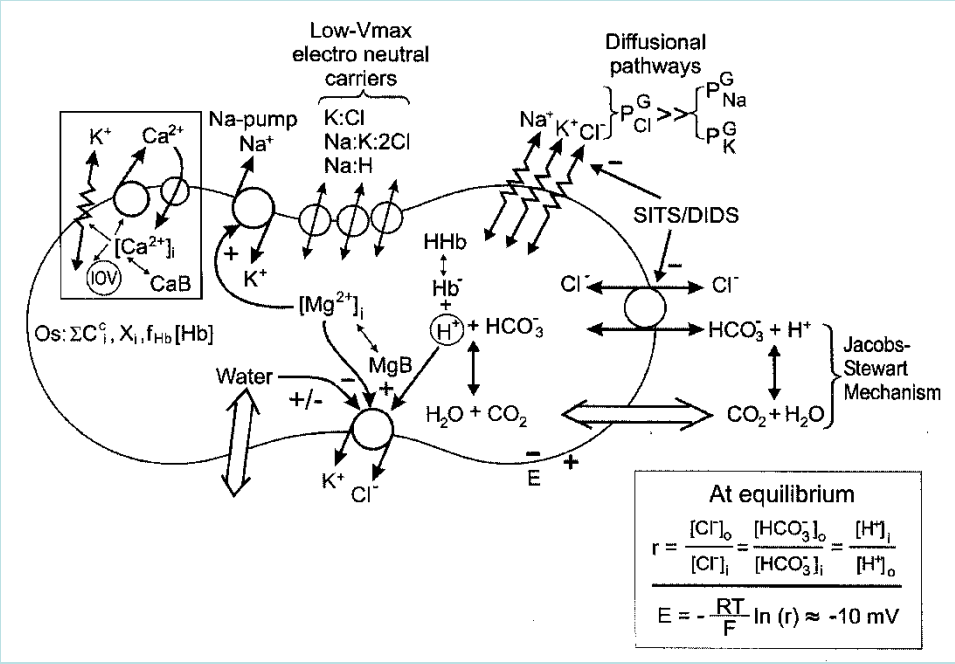




Conclusion: ~ 80% pellet reduction is by spontaneous vesiculation

A bit of background…

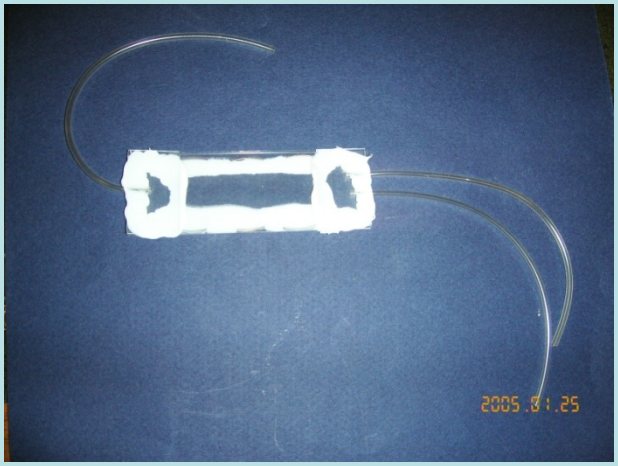
Main membrane transporters of human RBCs

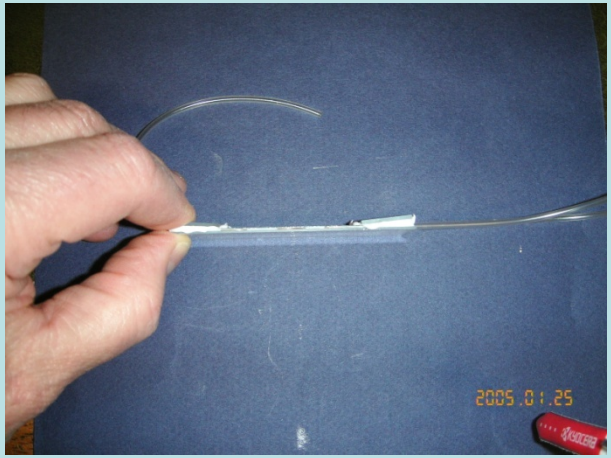


- **The main transport systems in human RBC membranes are**:

- *The anion exchanger (about 1.1 million copies/cell)*
- *The Ca pump (about 1000 copies/cell)*
- *The Na pump (about 400 copies per cell)*
- *The Gardos channels (Ca-sensitive, K-selective,* hIK1, hSK4, *about 200 copies/cell)*

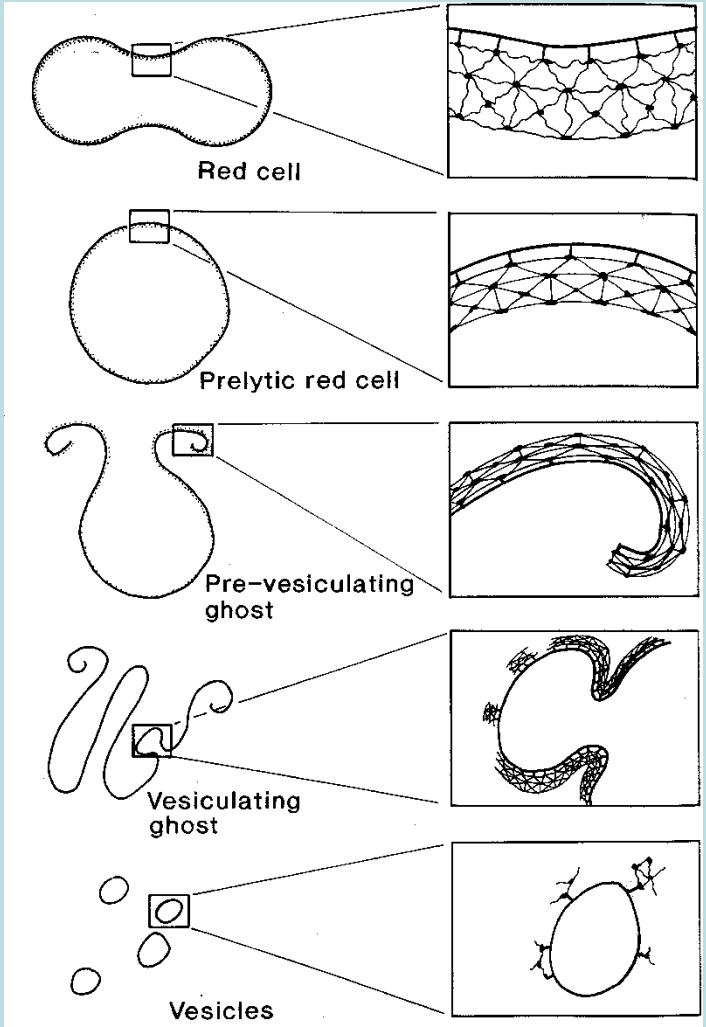
How we observed the vesiculation process





- A drop of the ghost suspension is placed between slide and coverslip
- The slide is designed as a temperature-controlled chamber by flowing water through
- At t=0 the temperature is increased from ~4^{o}C to ~37^{o}C in ~ 20s
- The membranes are observed under phase contrast at x1000
- Changes are recorded in real time

Cytoskeletal muscle



- The vesiculation conditions tense the cytoskeletal mesh
- Tension expands the lytic hole into a free, IO-curling edge
- The tension value in each ghost determines the ROG or IOG pre-vesiculation shape of the ghost and thus the subsequent vesiculation path
- Spectrin and actin fall off only at the final, vesicle sealing stage

What do the residual fibrillar projections tell us about vesicle sidedness and the mechanism of formation of concentric double and treble vesicles?

Expected from sequential endocytosis

Observed

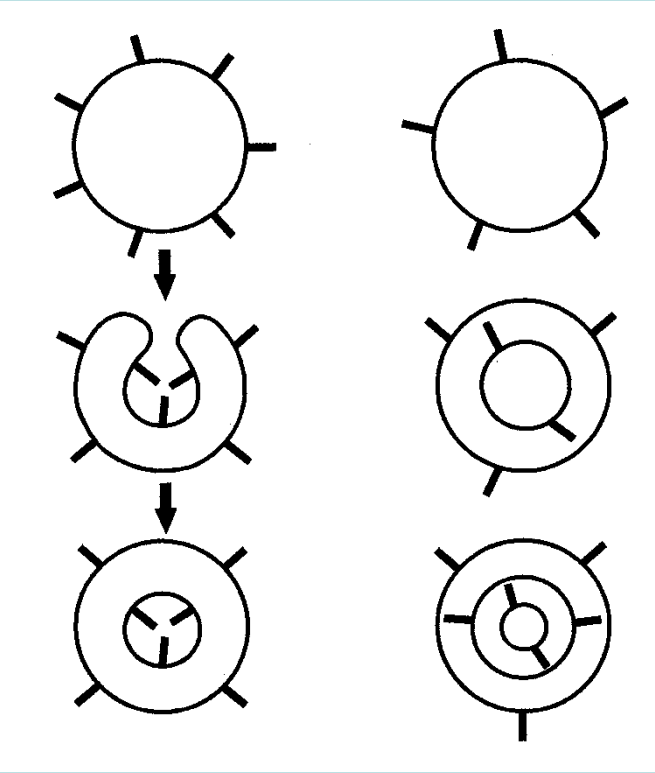


Table 1. Sidedness of Spontaneously Formed Red Cell Membrane Vesicles

Type of vesicle	Total number of each type	Membrane sidedness			Number of each form
Single	29	IO			24
		RO			3
		NC			2
		Outer	Inner		
Double	13	IO	IO		11*
		NC	NC		2
		Outer	Middle	Inner	
Treble	6	IO	IO	IO	5*
		IO	IO	NC	1

Membrane sidedness was observed in high magnification EM sections of vesicles formed after 20 min at 37°C. By this time the dense fibrillar projections which characterized initially the inner membrane surface (Fig. 1) had become more scattered (Fig. 3). Sidedness was determined by identifying residual projections attached to the inner or outer aspect of each concentric ring. IO, inside out; RO, rightside out; NC, not clear.

* Including vesicles in Fig. 3, *e* and *f*).

Can we attempt an explanation of the vesiculation process from serial section reconstructions?

The challenge

How can we apply the information obtained from the optical and EM studies to gain an understanding of the dynamic processes leading from ghost to bunch of vesicles in each cell

Example: serial sections from spiral to concentric circles

So, the membranes had to "do" somehow what I have done with scissors and tape in order to generate the serial section sequence observed

More background…

- SK-IOVs were prepared by lysing RBCs in divalent cation-free DW with very low concentrations of buffer to pH 7.5-8.0, followed by lengthy washes and cold-incubation steps lasting about two days.
- These IOVs preserved the function of all but one of the RBC membrane enzymes and transporters
- The function of the Kcnn4 Ca^{2+}-sensitive K^+ channel was missing
- ***In the process of searching at what stage in the SK-IOV procedure channel function was lost we discovered that IOVs can form spontaneously within minutes of RBC lysis, and that we could follow their formation under the microscope***
- These one-step IOVs did conserve Kcnn4 function and our initial studies focussed on this channel (Lew et al., Nature. 1982. 296:742-744)

But by far the most fascinating and challenging issue was trying to understand how these IOVs were formed

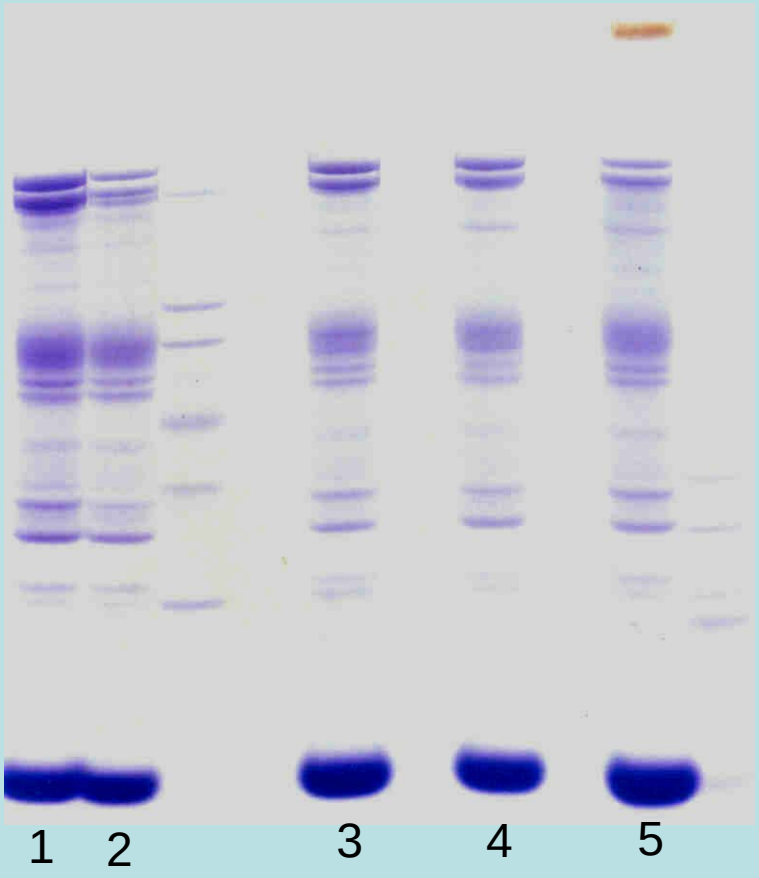
...a particularly nagging challenge because the images were showing membrane discontinuities considered thermodinamically impossible at the time, and an uninterpretable dynamics for IOV formation

Molecular model of spontaneous vesiculation: roles of cytoskeleton and lipid bilayer

- **Cytoskeleton**:
  - Disassembly of the cytoskeleton provides the energy and “muscle” for the membrane motions
  - The disassembly modality determines the vesiculation pattern in each ghost

- **Lipid bilayer**:
  - Special configurations of integral membrane proteins mediate free edge stabilization and cutting-splicing processes responsible for vesicular sealing

Divalent cations arrest vesiculation by crosslinking spectrin



- 1. Before vesiculation
- 2. After vesiculation
- 3. Mg^{2+} (reversible)
- 4. Ca^{2+}
- 5. Co^{2+}

- Co^{2+} cause extra spectrin depletion forming a high MW cross-linked protein which cannot penetrate the gel

- Crosslinking of spectrin stabilizes the cytoskeleton and prevents further disassembly