cytochrome oxidase. This is supported by evidence from model compound studies (*12*), in which only those compounds with weak *trans* ligands [for example, Tpiv PP THF (CO)] or no *trans* ligand at all [for example, Tpiv PP(CO)] have frequencies as high as 520 cm^{-1}. Also, the frequency of the Fe-histidine stretching mode in reduced cytochrome oxidase is 214 cm^{-1}, a value lower than that for all other proteins except the α chains of T-state deoxyhemoglobin, which is also proposed to have a destabilized iron-histidine bond (*17*). This frequency in the reduced enzyme is therefore consistent with a strained (weak) Fe-histidine bond. Finally, in low-temperature magnetic circular dichroism (MCD) studies, the photodissociated heme in cytochrome oxidase was found to have the same MCD spectrum as the fully reduced heme (*18*). In contrast, the spectra of photodissociated hemoglobin and myoglobin differ from that of the corresponding deoxy preparations. The MCD data were interpreted (*18*) as indicating that the histidine-heme complex is confined to the same conformation when six-coordinated as it does when five-coordinated, which is the conformation with the iron atom held out of plane. This interpretation is consistent with our discovery of a weakened Fe-histidine bond in the six-coordinate heme.

Our conclusions as to these structures (See Fig. 3) clarify some of the physiological properties of cytochrome oxidase binding to both oxygen and carbon monoxide. The affinity of MOX for CO is lower than that of myoglobin (*19*). We found that the bound CO is tilted in the MOX presumably because of an amino acid residue present in the distal pocket. This should result in a restricted and unfavored entry to the binding site for the CO and consequently in a low on-rate and low affinity. The measured on-rate for CO ($\sim 1 \times 10^5\ M^{-1}\ sec^{-1}$) (*19*) is lower than that of myoglobin ($\sim 5 \times 10^5\ M^{-1}\ sec^{-1}$)] (*20*). The off-rate, which results from a unimolecular dissociation, would be expected and is observed to be similar in MOX and myoglobin since the Fe-C bond strengths are comparable (that is, the stretching frequencies are both in the 500-cm^{-1} range). The steric interaction in the binding site would not affect the oxygen binding since it binds preferentially in a bent orientation.

The intrinsic affinity for oxygen binding to MOX is difficult to measure because the oxygen is reduced by the oxidase and the cytochrome a_3 is oxidized. Values for the association constant have been reported to be from 10^6 to 10^8 (*21*), although the results of low-temperature experiments have been interpreted (*22, 23*) as evidence for a lower value for the actual binding to the iron. The apparent high affinity under physiological conditions has been described as a consequence of secondary reactions that are subsequent to binding to the iron and culminate in the oxygen reduction (*23*). Our data on (CO)MOX indicate that the Fe-histidine bond is strained when the oxidase is six-coordinated. We interpret this strain as evidence that the iron movement into the heme plane is restricted upon oxygen binding and causes a weak iron-dioxygen bond. These data support proposals that oxygen binds only weakly to the iron in cytochrome oxidase and that the apparent high affinity for oxygen results from additional reactions.

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Mechanical Wounding Induces the Formation of Extensive Coated Membranes in Giant Algal Cells

Abstract. *Mechanically wounding giant cells of* Boergesenia forbesii *induces the formation of bristle-coated, plasma-membrane invaginations (coated pits) and coated vesicles, easily providing a plentiful source of coated membranes in a clean cellular system unencumbered by other tissues. Contractions evoked by wounding partition the cytoplasm into hundreds of spherical protoplasts with approximately 40 percent less total plasma-membrane surface area than the original cell. Ferritin labeling and the appearance of numerous large coated pits and vesicles at the peak period of contraction indicate that these organelles play a role in extensive endocytosis of the plasma membrane.*

Giant cells of plants (*1–3*), animals (*4*), and protists (*5*) heal wounds through the activity of putative contractile mechanisms (*3, 6*). In some plant cells (*1, 2*), being wounded induces cytoplasmic contractions that result in the complete segregation of cytoplasm into numerous spherical protoplasts within 90 minutes (Fig. 1, A to E). Ultrastructural studies of this phenomenon in the giant-celled green alga *Boergesenia forbesii* (*7*) reveal that approximately 45 minutes after wounding, extensive coated pits and vesicles become evident in and near the plasma membrane (Fig. 1F–H). They continue to be abundant as long as 75 minutes after wounding, whereafter they are only infrequently observed. The 30-minute interval of their observed occurrence corresponds to the peak period of cytoplasmic contraction that leads to protoplast formation (*2*). The average sizes of coated pits and coated vesicles are 250 nm (range, 80 to 625 nm) and 175 nm (100 to 350 nm, outside diameter), respectively. This variability in size is

typical of such structures in algae, but the dimensions are relatively large (*8*). The coating can be seen in considerable detail, even with standard preservation techniques, and it resembles the typical protein (clathrin) coat reported for these organelles in various cell types (*8*). Grazing sections reveal the characteristic polygonal pattern (Fig. 1I); each polygon has a 26-nm diameter, and the sides of the polygons are 11 nm wide. Longitudinal sections through the coat show that the sides of the polygons have an average height of 26 nm (15 to 44 nm) and an average spacing of 20 nm at their distal ends. Cells wounded in the presence of ferritin (*7*) exhibit similar coated membranes, with the ferritin lining the pits and filling the lumen of the coated vesicles (not shown). Preliminary evidence indicates that the ferritin is eventually deposited in nascent vacuoles throughout the peripheral cytoplasm of the protoplasts.

Table 1. Total plasma-membrane surface area in five sizes of *Boergesenia* cells before and after segregation of the cytoplasm into hundreds of tiny spherical protoplasts.

Cell	Total plasma-membrane surface area (mm^2)		Decrease (%)
	Intact cell	Protoplasts	
1	29.61	13.52	54.3
2	14.98	9.76	34.8
3	21.73	12.88	40.7
4	26.06	15.03	42.3
5	53.26	37.03	30.2
Mean			40.5

The simple geometry of intact cells and of the resulting protoplasts permits quantitative analysis of surface areas with an image-analysis computer system (Zeiss IBAS) (*9*). The total plasma-membrane surface area of intact cells and that of the protoplasts resulting from wounding *B. forbesii* cells appear in Table 1. On the average, the total plasma-membrane surface area of the protoplasts formed from a single wounded cell is 40 percent less than that of the original cell before being wounded. This dramatic decrease in total plasma membrane is correlated with the appearance of coated membranes in wounded *Boergesenia*, and it implicates their role in the endocytosis of a considerable quantity of excess plasma membrane. Cytochemical evidence from other investigators reveals that coated pits and vesicles function in endocytosis in many cell types (*10*). Ferritin labeling and quantitative analysis in this study, which correlate coated-membrane formation with the decrease in the amount of surface membrane, also support this role in *Boergesenia*. This extensive endocytosis apparently occurs without the complementary exocytosis reported in a few other organisms (*11*). Also, the scarcity of extracellular membranes or vesicles observed indicates that virtually no membranes were lost to the exterior during protoplast formation.

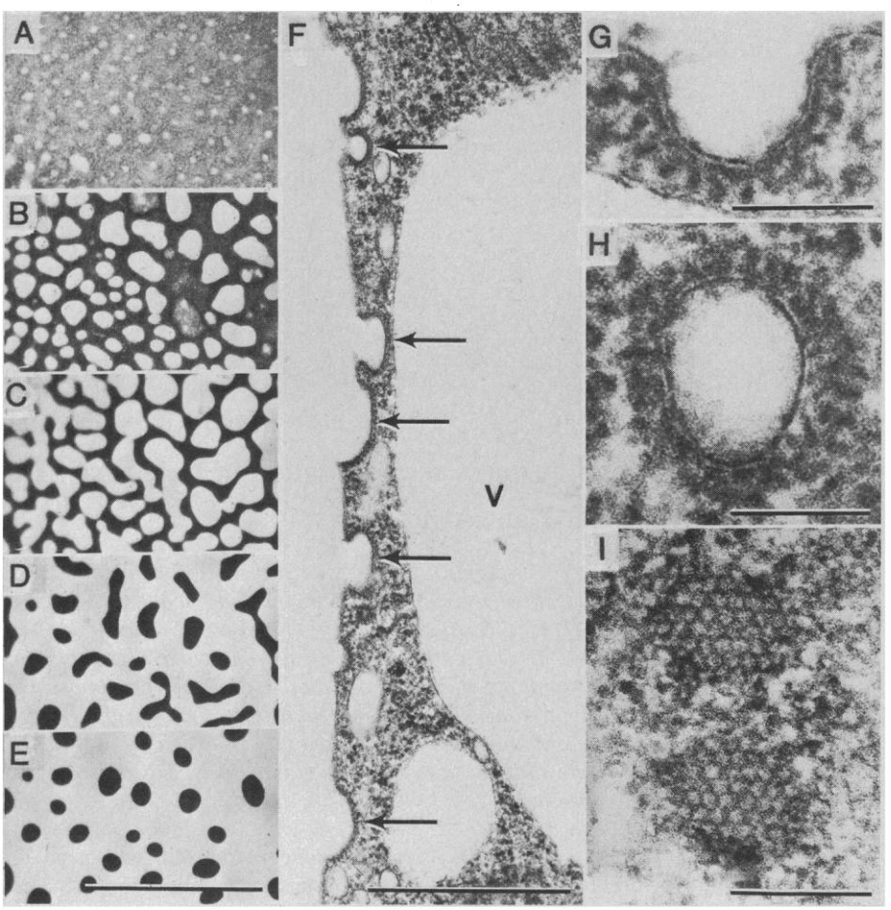

Fig. 1. Cytoplasm in surface view of *Boergesenia forbesii* (A) 30, (B) 45, (C) 60, (D) 75, and (E) 90 minutes after wounding. Note the aggregation of the cytoplasm into many spherical protoplasts within 90 minutes of puncturing the cell with a needle or making a small incision in one end of the cell. (F to I) Electron micrographs of cells fixed about 60 minutes after being wounded, showing longitudinal sections at or near the plasma membrane. (F) Numerous large coated pits occur in the plasma membrane (arrows), invaginating into the thin cytoplasmic layer between the cell wall (out of view to the left) and the large central vacuole (V). Higher magnifications of a coated pit (G) and a coated vesicle (H) revealing the distinctive coating on the cytoplasmic surfaces of the membranes. Tiny discontinuities in the membranes may represent clathrin attachment sites. (I) Grazing section of two coated vesicles, revealing the polygonal pattern of the clathrin-like coat. Scale bars: (A–E) 1.0 mm, (F) 1.0 μm, (G–H) 0.1 μm, and (I) 0.2 μm.

The functional multiplicity of clathrin-coated pits and vesicles is apparent from the literature. They play an endocytic role in receptor-mediated uptake of substances, in recovery and recycling of Golgi membranes, and in pinocytosis (*8, 10, 12*). We believe that coated pits and vesicles in *B. forbesii* may retrieve excess plasma membrane during protoplast formation, since their sudden appearance is temporally and spatially correlated with cytoplasmic contractions that lead to the marked decrease in total plasma membrane. Our findings differ from those reporting a thousand-fold increase in surface area during cyst formation in the green alga *Acetabularia*, in which coated vesicles function in exocytosis (*13*). This apparent discrepancy can be explained: cyst formation in *Acetabularia* involves complex cytoplasmic cleavages that require additional membranes (*13*) instead of the substantial cytoplasmic contractions leading to protoplast formation in *Boergesenia*.

To our knowledge, this is the first report of "mechanical" inducement of coated-membrane formation in any organism, although physiological (chemical) induction has been investigated in several other systems (*10, 14*). The only requirements of contraction in *Boergesenia* and related algae are metabolic energy and micromolar amounts of free Ca^{2+} (*3, 6*). The contractile function of

the extensive cytoskeletal system in these algae (*15*) suggests several possibilities in using *B. forbesii* for studying the functions of microtubules and microfilaments in endocytosis and the development of coated membranes. The size and abundance of coated organelles, combined with their easily inducible and predictable occurrence, make this unusual alga a model system for isolating and characterizing coated membranes.

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7. Cells were fixed with 0.5 percent glutaraldehyde and 0.2 percent osmium tetroxide in cacodylate buffer containing salts to give a final tonicity of 900 mosmol/kg, *p*H 7.4, for 10 minutes at room temperature. After rinsing and desalting, cells were stained en bloc with 0.5 percent uranyl acetate for 60 minutes on ice. Acetone dehydration was followed with infiltration and embedment in Epon-Araldite. Sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (JEOL JEM-100CX) at 80 kV. Ferritin labeling was accomplished by wounding cells in 5 ml of growth medium (*2*) containing 2.3 mg of ferritin per milliliter (Miles-Yeda, cationized, lot CF8) at room temperature. After 60 minutes the cells were washed twice with growth medium and fixed as above.
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9. Photographs of entire intact cells and of the same cells after protoplast formation were analyzed. After the photographs were video-digitized, image enhancement removed protoplasts below the upper focal plane in the photographs, permitting analysis solely of the upper half of each cell (that portion in sharp focus). Absolute values in square millimeters were obtained by incorporating original magnifications of micrographs into the program. The initial cells chosen were nearly cylindrical, so the planar area determined by the computer was multiplied by π to give the total plasma-membrane surface area in the intact cells. This value for cell 2 in Table 1 was close to the value calculated through direct geometrical analysis (by hand) of the micrograph (14.57 mm^2), verifying accuracy of the program and of our assumptions in the procedure. The protoplasts resulting from wounding are nearly spherical, so their two-dimensional areas (πr^2) were summed for each cell by the computer and multiplied by 4 to give the total surface area. This value was then doubled, since only half of each cell was in sharp focus (due to their large size) and therefore analyzed. Accuracy of the protoplast values was checked mathematically by measuring the diameter of each of the protoplasts that formed after wounding and calculating the total surface area knowing that no cell wall is present yet and that each has a smooth surface membrane (unpublished observations). These values were consistently lower than those calculated by the computer, indicating that the decreases (Table 1) are conservative.
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Adsorption to Fish Sperm of Vertically Transmitted Fish Viruses

Abstract. *More than 99 percent of a vertically transmitted fish rhabdovirus, infectious hematopoietic necrosis virus, was removed from suspension in less than 1 minute by adsorption to the surface membrane of sperm from two genera of salmonid fishes. The vertically transmitted, infectious pancreatic necrosis virus adsorbed to a lesser degree, but no adsorption occurred with a second fish rhabdovirus that is not vertically transmitted. Such adsorption may be involved in vertical transmission of these viruses.*

In a review of vertical transmission of viruses, the role of semen in viral transmission was pointed out as being largely neglected by virologists (*1*). Most studies of mammalian viruses in semen have dealt only with venereal (horizontal) rather than vertical transfer (*2*). Investigators have rarely discriminated between virus in semen unassociated with sperm (*3*) and viruses within the sperm cell (*4*), but surface adsorption of virus to sperm cells has been described only for simian virus 40 with sperm from a rabbit, an atypical host (*5*). In oviparous species with external fertilization, venereal infection by viruses in semen is less likely to occur than vertical transmission. In vertical transmission, virus closely associated with the sperm would be more efficiently transferred to the egg than unassociated virus. We now report evidence that a vertically transmitted fish rhabdovirus adsorbs rapidly to fish sperm, whereas a closely related rhabdovirus not vertically transmitted does not.

Infectious hematopoietic necrosis (IHN) is a common rhabdoviral disease of salmonid fishes found on the west coast of North America and can be transmitted both horizontally (from fish to fish) and vertically (from generation to generation). The disease has occurred in fish in other parts of the continent, apparently associated with the shipment of eggs from infected fish (*6*). Both the history of the disease and the characteristics of the virus have been reviewed (*7*). To determine whether IHN virus adsorbs to sperm and thus whether it is involved in vertical transmission, we obtained milt from several steelhead (anadromous rainbow) trout (*Salmo gairdneri*) and pooled them (6.8×10^9 sperm per milliliter); the pooled milt was centrifuged, the seminal plasma was decanted and saved, and the sperm cells were washed twice with diluent (cell culture medium without fetal bovine serum). The milt was assayed for the presence of IHN virus; no virus was found. The sperm was then divided into equal portions and centrifuged, and 1 ml of an IHN virus suspension was added to each of six sperm pellets and to each of six control tubes containing a volume of diluent equal to the volume of sperm. The mixtures were incubated at 15°C for 1 hour with continual agitation and then centrifuged, and the virus remaining in the supernatants was measured by a plaque assay (*8*). The presence of sperm reduced the amount of virus remaining in the supernatant by 99.6 percent compared to controls (Table 1).

To determine whether reduced virus counts were due to inactivation by residual seminal plasma, we sterilized by filtration the fluid recovered from the initial centrifugation of the milt and added it to a suspension of IHN virus at a ratio of 9 to 1 (by volume). Control tubes received an equal volume of diluent. After 1 hour the amount of virus remaining in the supernatants was measured. Seminal plasma did not inactivate the virus (Table 1). For all further work, the seminal fluid was not removed from the sperm.

Cavity fluid is a viscous liquid expelled by female fish along with the eggs and can contain high concentrations of IHN virus. We tested its influence on adsorption of virus to sperm by adding cavity fluid from virus-free steelhead trout to IHN virus at a ratio of 9 to 1 (by volume). Sperm was added to one group of tubes, and diluent was added to control tubes. After incubation, more than 99 percent of the virus had been removed