Chapter 3

ADHESION OF FOULING DIATOMS TO SURFACES: SOME BIOCHEMISTRY

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3.1 INTRODUCTION

In spite of the fact that most studies of microbiological fouling show the presence of diatoms on fouled surfaces, little is known of the contribution of these organisms to the ecology of the biofilm. A cursory assessment of the microfouling literature may suggest that bacteria represent the only fouling problem. While that may be true for surfaces that are dark, it certainly does not apply to the lighted surface. All substrata that are wet and illuminated ultimately will support the growth of an algal population. In many cases these algae will be diatoms. Diatoms may play a considerable role in the nutrient dynamics of the biofilm (Characklis and Cooksey, 1983; Escher and Characklis, 1982) through syntrophic interactions. Such interactions are well known for the water column (Bell and Sakshaug, 1980; Brock and Cline, 1984) where the syntrophic partners are much further apart and diffusion paths much longer than in a biofilm. Cross-feeding in biofilms may involve much more than exchange of organic molecules. It could, for instance, involve the detoxification of the biofilm environment by bacterial respiration of photosynthetically-produced oxygen.

Before diatom attachment takes place, the cells must be transported to a surface. In all but totally quiescent situations this process occurs by hydrodynamic means (Breznak et al., 1985).

It is the purpose of this paper to throw some light on some of the metabolic events which take place immediately following the arrival of a raphe-bearing diatom on a surface.

3.2 METHODS

3.2.1 Choice of organism

Except where stated otherwise, the experimental organism used in this study was Amphora coffeaeformis (Agardh) Kutz (Fig. 3.3). A. coffeaeformis grows heterotrophically and mixotrophically on glucose (Cooksey and Chansang, 1976).

3.2.2 Electron microscopy

This has been described (Webster et al., 1985).

3.2.3 Video recording

Video recordings were made in the β-III mode on a Toshiba recorder (Model V8500T). Images were obtained from a Reichert phase-contrast-microscope fitted with an RCA video camera (Model TC2011). The video image was modified to contain a time/date signal (RCA Model TC1440B). The overall magnification was 330x (microscope 16x, electronic approximately 20x).

3.2.4 Chemotaxis Experiments

Two methods were used. In the first, sterile 90 mm Petri dishes were filled with artificial seawater medium (Provasoli et al., 1957), modified to contain 5 mM Ca^{2+} and 2% agar (Cooksey and Chansang, 1976) to a depth of 4 mm. A well was cut in the agar which was then filled with sterile 10 mM glucose in the same medium. After incubation at 28°C overnight, diatoms were applied to the surface of the agar in a line 20 mm from the glucose-containing well. A fine, sterile paint brush which had been dipped in an axenic diatom suspension was used for this operation. Nineteen hours from the addition of the glucose to the agar, the plates were observed as described above. Cells moving up or down the glucose gradient were identified by means of the tracks left in the agar surface and counted. In some experiments, tracings of the tracks were made and used for measurements of changes in direction in response to the glucose gradient in the agar.

A second method for the detection of chemosensing of glucose was based on that of Zigmond (1977). In this procedure diatoms attached to a glass microscope cover-glass were exposed to a chemical gradient established between two wells in an acrylic plastic microscope slide. Again cells moving up or down the gradient and travelling at an angle of no more than 45° to the gradient were counted. This represented a highly conservative assessment of orientation. Results were calculated with respect to a normal approximation of a binomial distribution with $P<0.05$ and compared to a theoretical distribution of 50% positive orientation.

3.2.5 Incorporation of radio-labelled substrates

Details are given in each experiment. The general procedure has been published (Cooksey, 1978). Where drugs were used, control incubations contained the same concentration of solvent as the drug-containing incubations.

3.2.6 Adhesion assays

These were carried out as described earlier (Cooksey, 1981).

3.2.7 Preparation of diatom footpads

Cells were attached to glass microscope cover glasses as described (Cooksey, 1981). The cover glasses were rinsed to remove unattached cells and placed in 10 mM ethylene glycol bis (β-aminoethylether) N,N^1-tetraacetic acid (EGTA) in minimal media less calcium at pH 7.7 for 20 min. at room temperature. They were then rinsed further with minimal medium and observed microscopically using phase-contrast optics.

3.2.8 Drugs, inhibitors, radiochemicals and counting procedures

3-(3,4-dichlorophenyl)-1,1- dimethylurea (DCMU) was obtained from K&K Laboratories Inc., and recrystallized from aqueous ethanol before use. α-isopropyl-α-[(N-methyl-N-homoveratryl)-α-amino propyl] -3,4,5- trimethoxy-phenylacetonitrile (D-600) was a gift from Knoll A. G., West Germany. Carbonyl cyanide -3-chlorophenyl hydrazone (CCCP), cycloheximide (CH) and tunicamycin were obtained from Sigma Chemical Co., USA, and used without further purification. All drug solutions were made in dimethylsulfoxide or 70% (V/V) ethanol at such a concentration to allow no more than 1% of the solvent in the incubation mixture. ^{3}H-glucose was obtained from ICN Corporation, USA. Radioactivity was determined in cells after solubilization in Protosol and counting in Aquasol II (New England Nuclear Corporation, USA).

3.2.9 Cell counts

Diatom cells were counted in a haemocytometer. Viable cells were counted after treating with Trypan blue (final concentration, 0.5%). Unstained cells were considered viable.

3.3 RESULTS AND DISCUSSION

A. coffeaeformis has been shown to respond to a glucose gradient in agar by moving up the concentration gradient (Table 3.1). In the absence of glucose, cells move in a series of random ellipses. The cause of the elliptical movement is probably the geometry of the raphe systems. A. coffeaeformis possesses two raphes on the ventral side of the organism. It has been suggested (Cooksey and Cooksey, 1986) that one of the raphes is in closer contact with the substratum than the other and thus transmits the 'dominant' driving force. When in a gradient, however, A. coffeaeformis moved in a series of separate slightly curved lines, each of which followed a direction change so

TABLE 3.1

Chemosensing in a glucose gradient in agar.

Expt. No.	Total cells counted	Percent moving towards glucose ± (S.E.)[a]
1	31	90 ± (66 - 99)
2	38	92 ± (74 - 99)
3	50	92 ± (77 - 99)
4	82	77 ± (63 - 88)
5	74	85 ± (72 - 94)
6	48	71 ± (51 - 86)

[a]Results significant at $P<0.01$.

as to maintain movement along the gradient (Fig. 3.1). It is possible to regard undirected movement as a series of error accumulations with respect to an imaginary straight line. On the other hand, directed movement as a result of the repeated sensing of a chemical gradient, results in constant error correction and implies that at least in this organism differential raphe control is possible.

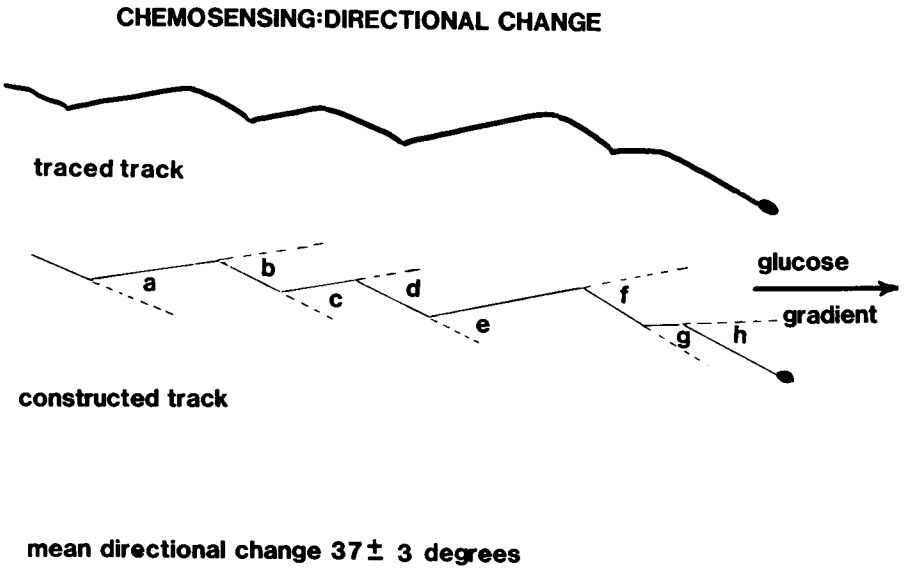


Fig. 3.1. Directional Changes in Response to a Glucose Gradient. (i) The tracks of a diatom on an agar surface were traced from the video monitor. (ii) The series of curves in (i) were straightened in order to measure the angle changes made by the diatom. The mean angle change (a - h) was 37° ± 3°.

Although the agar plate method was particularly suitable for the observation of the behavioral responses of the cell to a glucose gradient, it was less so for the quantification of the concentrations of chemoattractant necessary to elicit a response. For this work we chose to use a Zigmond chamber - an apparatus very convenient for the observation of slow-moving organisms, i.e. those that move at a few μm/sec. Fig. 3.2a shows the results of a time-course with 0.5 mM glucose. After random movement at first, cells become oriented after 20 min so that 70% are moving up the gradient. At present we do not know whether the lag in response to glucose is caused by the time needed for the gradient to be established, or the time necessary to induce a metabolic response to glucose. Glucose uptake and metabolism is an inducible property of this organism that normally takes 25 min to develop (Cooksey, unpublished observation). It will be informative to discover whether glucose-induced cells also show this lag in orientation response. Should such an induction period be necessary to sense all extracellular chemical signals, it would imply that a time of this order would be necessary for the cell to adhere as a result of such a signal. This seems unlikely to us. The minimal glucose gradient detected was 0-0.1 mM glucose which was established over 2.5 mm distance. This translates to an approximate gradient of 0.04 μM/μm of cell length. In control experiments, where no glucose is present cells moved randomly (Fig. 3.2b), i.e. orientation was not different from 50%.

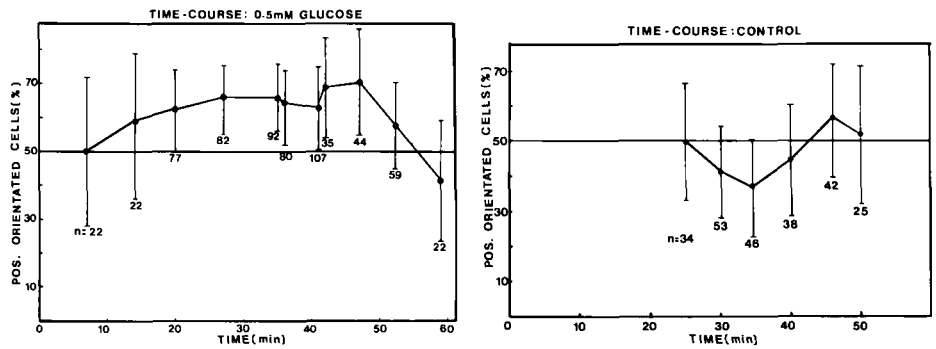


Fig. 3.2. (a) Time-course for the Development of Cell Orientation in a Glucose Gradient. Diatom cells on a microscope cover-glass were exposed to a 0-0.5 mM glucose gradient in a Zigmond diffusion chamber. Total number of cells (n) moving up the glucose gradient (pos.) are expressed as a function of the total number of cells moving up and down the glucose gradient, together with the 95% confidence intervals ($P<0.05$), (b) a similar time-course in the absence of a glucose gradient.

The physiological requirements for the adhesion of A. coffeaeformis to glass were investigated by means of the quantitative adhesion assay. We found that Ca^{2+} or Sr^{2+} were necessary with no other ion tested being able to substitute (Table 3.2). It should be noted that although cells would adhere in the presence of Sr^{2+}, they were not able to move, whereas cells that adhered in the presence of Ca^{2+} were motile (Fig. 3.3b). Further, cells adhered in Sr^{2+} could be distinguished from those in Ca^{2+} by the morphology of the adhesive polymer (Webster and Cooksey, unpublished observation). This implies that Sr^{2+} can substitute for Ca^{2+} structurally, but not in the secretion process (Cooksey et al., 1984). The response to Ca^{2+} was not an isolated one and was found with several other pennate fouling diatoms (Table 3.3). Adhesion took place in the light or dark and was not inhibited by the photosystem II inhibitor DCMU.

TABLE 3.2

Adhesive Response of A. coffeaeformis to the Presence of Divalent Cations

Ion	Percent of Control[a]
Control, 0.25 mM Ca^{2+}	100 (36)
5 mM Ca^{2+}	354 ± 69 (12)
5 mM Sr^{2+}	193 ± 4 (6)
10 mM Sr^{2+}	427 ± 14 (6)
5 mM Mn^{2+}	60 ± 27 (6)
5 mM Ba^{2+}	0 (6)

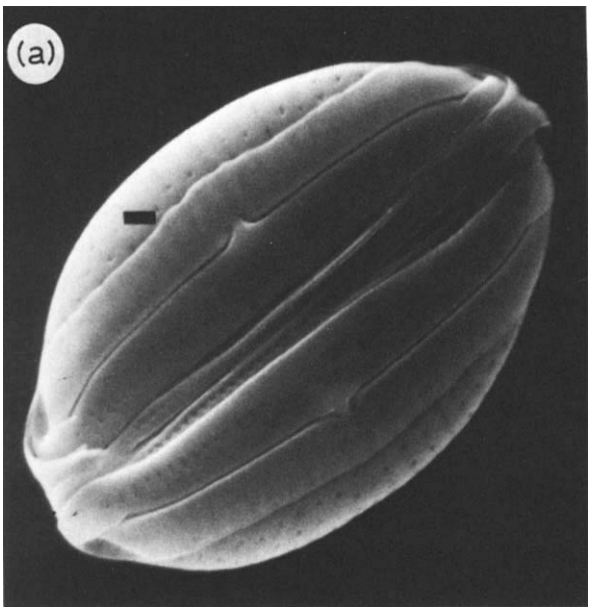
[a] Adhesion of diatom cells to a glass surface was measured by the method of Cooksey (1981). Figures are means ± 1 standard deviation (no. of determinations). Data from Cooksey, 1981.

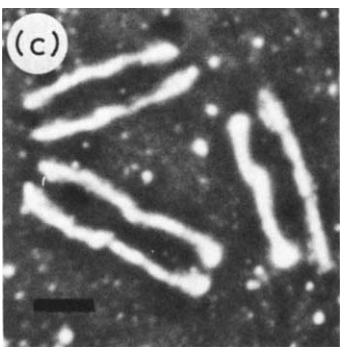
TABLE 3.3

Adhesion to Glass by Marine Fouling Diatoms Grown in Artificial Seawater Medium at Various Concentrations of Calcium

Organism number	4	6	25	27
Genus	Navicula	Nitzschia	Tropidoneis	Nitzschia
Cell length (μm)	10	13	23	50
Adhesion in ASP-2				
medium + 0.75 mM Ca	-	+/-	-	+
2.5 mM Ca	+	-	+/-	+
10.0 mM Ca	++	+	++	++

-, no adhesion; +/-, gentle agitation suspends cells; +, shaking suspends cells; ++, shaking does not remove cells from glass. Experiment carried out in 5 ml portions of ASP-2 medium (Provasoli et al., 1957) at the Ca concentrations shown.





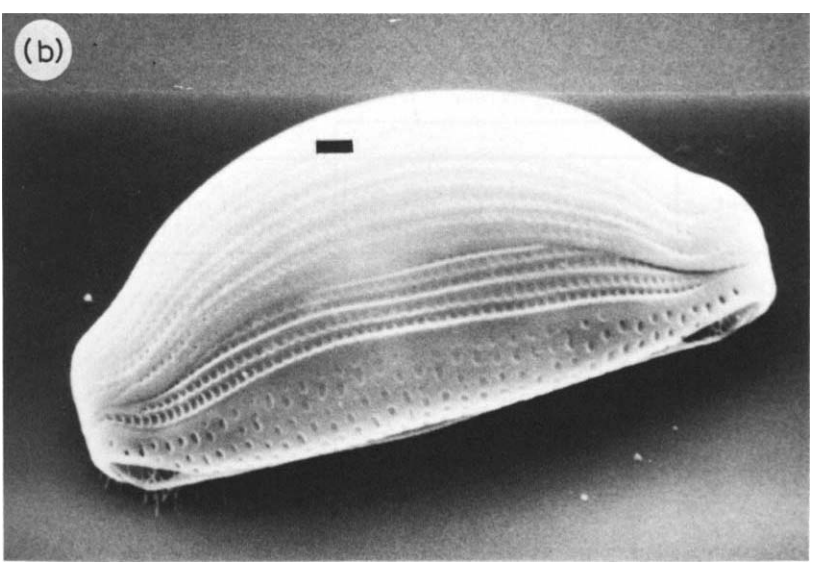


Fig. 3.3. (a) SEM Photomicrograph of Cleaned Frustule of A. coffeaeformis. Ventral view showing raphe openings. Bar = 1μm. (b) A. coffeaeformis adhered to glass surface. Note position and size of adhesive appendages. Bar = 1μm. (c) Phase-Contrast Micrograph of Raphe Imprints of A. coffeaeformis. Bar = 5μm.

It was inhibited by an energy uncoupler (CCCP), an inhibitor of protein synthesis (cycloheximide) and a Ca^{2+} transport inhibitor (D-600) (Table 3.4). These results imply that energy is required for adhesion, i.e. it is not a passive process and the cells are not inherently sticky. Further, protein synthesis is necessary. We are aware that CH has several other modes of action (discussed in Cooksey, 1981 and McMahon, 1975), but the fact that in this system the same very low level of drug (3.6 μM) causes an 84% inhibition of leucine incorporation into the hot trichloroacetic acid insoluble fraction of the cell (protein and glycoprotein) tends to support this observation (Table 3.5). D-600 acts outside the cell membrane (Mayer et al., 1972; Meisheri et al., 1981) and prevents Ca^{2+} entry, thus suggesting an intracellular role for Ca^{2+}. Such a role could be in the secretory, exocytotic process known to be involved in transport of the adhesive to the raphe canal (Webster et al., 1985). The proposal that Ca^{2+} acts internally does not rule out an external role for Ca^{2+} also. In fact, the results from the EGTA experiments do imply an external role. Footpads or raphe imprints were obtained after removal of Ca^{2+} with EGTA, a Ca^{2+} chelator (Fig. 3.3c). A suggestion of how these imprints could be made when a cohesive break in this adhesive material occurs as a result of the removal of Ca^{2+} is given in Fig. 3.4. Raphe imprints such as these have also been found by Daniel et al. (1980) using the SEM after mechanical removal of the diatom cells from the substratum.

TABLE 3.4

Inhibition of Adhesion of *A. coffeaeformis*.

Treatment	% of Control ± S.D. (No. of Determinations)
Control, 0.25 mM Ca, light	100
5 mM Ca, light	478 ± 168 (18)
5 mM Ca, dark	543 ± 156 (6)
5 mM Ca, light, + 2 μM DCMU	549 ± 122 (6)
5 mM Ca, light, + 5 μM CCCP	76 ± 4 (6)
5 mM Ca, light, + 3.6 μM CH	39 ± 13 (6)
5 mM Ca, light, + 25 μM D-600	55 + 50 (9)

Washed cells were suspended in minimal medium and preincubated for 15 min. (CCCP, DCMU, D-600) or 30 min. (CH), before Ca was added at the concentrations shown. Adhered cells were quantified as described (Cooksey, 1981). Data from Cooksey, 1981.

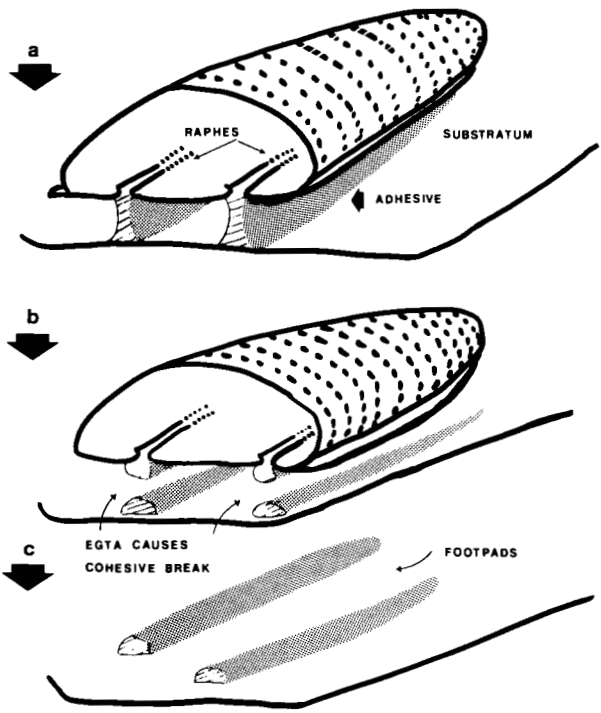


Fig. 3.4. Possible mode of action of EGTA in causing diatom removal from a surface.

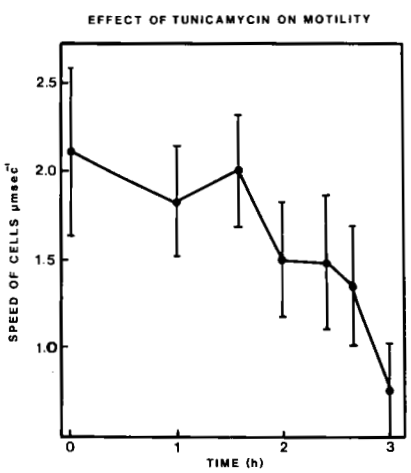


Fig. 3.5. Effect of Tunicamycin on Motility of A. coffeaeformis. Tunicamycin (0.5 μg/ml) was added to a suspension of diatoms in minimal media containing 5 mM Ca^{2+}. Speed of motility was measured from a videotape.

TABLE 3.5

Inhibition of Adhesion and Labeling of Hot Trichloroacetic Acid Insoluble Fraction of A. coffeaeformis.

Treatment	Percent Inhibition		
		Incorporation	
	Adhesion	Leucine	Glucose
Tunicamycin, 5 µg/ml	98	0	88
Cycloheximide, 3.6 µM	92	84	N.D.

N.D. = not determined.

The fact that the cytochemical work of Daniel et al. (1980) showed the diatom adhesive polymer to be an acid polysaccharide and our subsequent finding that an inhibitor of protein synthesis prevented adhesion, suggested that we investigate the effect of an inhibitor of glycoprotein synthesis (Tamura, 1982) on adhesion. Tunicamycin (TM) prevented the adhesion of diatom cells (Table 3.6) and also at a low concentration slowed motility (Fig. 3.5). It was noteworthy that even high levels of TM did not affect cell viability (Table 3.6). That TM was affecting glycoprotein synthesis rather than protein synthesis is shown by Table 3.5. Here TM inhibits the incorporation of glucose but not leucine into the protein and glycoprotein fraction of the cell.

TABLE 3.6

Effect of Tunicamycin on the Adhesion and Viability of A. coffeaeformis.

Treatment	Adhesion (Percent Control Value)	Viability[a]	Motility
Control, no drug	100	79+9 (4)	all
0.5 g/ml tunicamycin[b]	5 (2)	N.D.	jerky
1.0 g/ml "	81±12 (16)	83±5 (4)	few
5.0 g/ml "	52±39 (14)	83±2 (4)	none
10.0 g/ml "	6±1 (7)	N.D.	none

[a] Viability as measured by the Trypan blue method.
[b] 24 h preincubation.
N.D. = not determined.

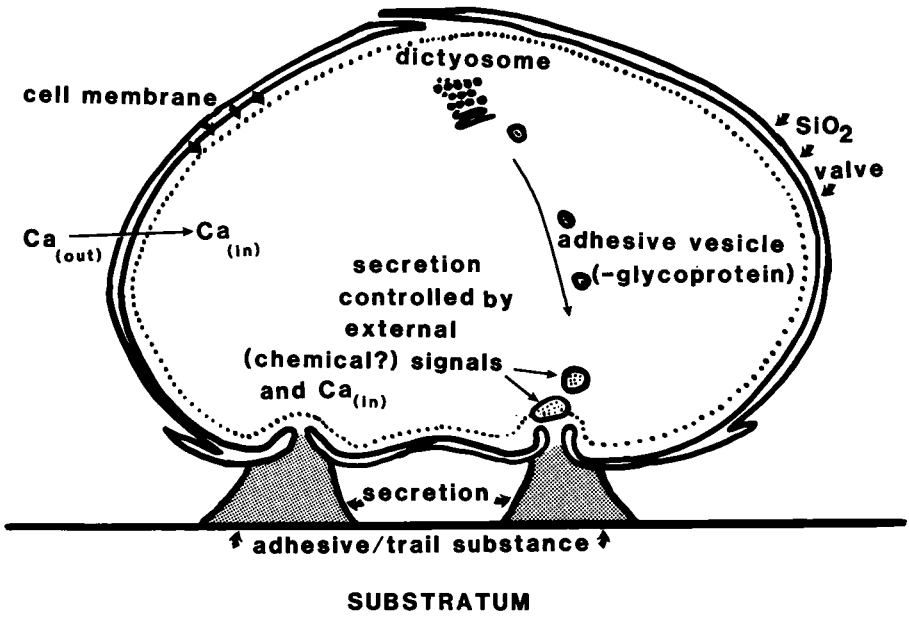


Fig. 3.6. A Conceptual Model of Pennate Diatom Adhesion.

The results presented here, together with the work of Chamberlain's group (Daniel et al., 1980; Daniel and Chamberlain, 1981) suggest a conceptual model for the steps in diatom adhesion (Fig. 3.6). Vesicles produced in the dictysome of the Golgi apparatus contain polysaccharide-like material (Daniel et al., 1980). These vesicles are transported to the cell membrane near to the raphe canal by some as yet unknown means, but microtubules may be implicated (Webster et al., 1985). Fusion of the vesicles with the cell membrane allows secretion of their contents. This material (Fig. 3.3b), which is still associated with the cell membrane, allows the cell to adhere to the substratum (Webster et al., 1985; Edgar and Pickett-Heaps, 1983). The inhibition of adhesion by TM and the possible secretion of a molecule with different rheological properties at low TM concentrations suggest the involvement of a glycoprotein in the synthesis of the adhesive. It has been suggested that cellulose is synthesized on a glycoprotein primer (MacLachlan, 1982). Perhaps a similar situation exists with respect to the diatom adhesive. Calcium is obviously involved at an intracellular and extracellular site(s). Intracellularly it may be involved in the secretory process and transport of vesicles; extracellularly it could be involved as a cross-linking agent between

localized negative charges. Which of the previous steps is controlled by sensing of an external chemical signal is not yet known, but it seems clear that such a control phenomenon is possible. Whether the potential chemical signal is processed directly or after intracellular metabolic conversion of the chemical is not known. Also unknown is the role of the cycloheximide-sensitive process. Perhaps this could involve the induced synthesis of a protein as a result of a chemosensory event. These aspects of the work will form the basis of further investigations.

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