Wax Microchannels in the Epidermis of White Clover

Abstract. *The application of conventional electron microscopy to the study of plant leaves and fruit readily displays the surface wax deposits, but not the wax pathways from the underlying cells. A modified freeze-etch technique shows both at the same time and indicates that wax is transported from the epidermal cells to the leaf surface in microchannels.*

The surface wax deposits on plant leaves and fruit have often been examined by electron microscopists using various replica techniques, but the paths by which the wax reaches the surfaces from the underlying cells have been difficult to demonstrate (*1*, *2*). There are two conflicting views: Bolliger's (*3*) study of angiosperms indicated that lipoid droplets moved through the cell wall to the outer surface by diffusion, but Scott and her co-workers (*4*) have with light microscopy observed wax canals in the epidermis of orange rind, in the onion leaf, and in the seed of *Cerdidium*.

These canals have not been demonstrated with the electron microscope in conventionally sectioned material. This is probably due to the absence of a canal-bounding membrane and the inability of usual fixatives and stains for electron microscopy to give contrast between the wax and its pathways, on the one hand, and the cell wall and cuticle on the other. Whether this is the reason or not, the recent development of the freeze-etch technique by Moor and Muhlethaler (*5*) offers an alternative means of investigation. I have applied a modified technique to leaves of several different plant species, but in particular to *Trifolium repens*. The modification made it possible to view the adaxial surface complete with its wax coating and at the same time to have a cross-sectional view of the cuticle and cell wall.

A mature clover leaf was cut into pieces approximately 1.5 mm square, and these were floated overnight, adaxial surface uppermost, on a 40 percent glycerol solution. Taking care that the upper surface remained free of glycerol, I placed one of these pieces on the surface of a droplet of glycerol solution on a copper disk. After the piece was rapidly frozen in liquid freon at −150°C, I transferred it to a Balzers Freeze Etch unit in the manner described by Moor *et al.* (*5*). The surface of the frozen leaf was lightly planed with a microtome blade held at −190°C until part of the leaf epidermis was removed. Then the specimen was etched for 2 minutes, shadow casting was performed at an angle of approximately 30° with platinum-carbon, and the specimen was coated from above with a film of carbon.

After thawing, the leaf tissue was floated on water in a petri dish, replica uppermost. The water was slowly replaced with fuming nitric acid, and the petri dish was transferred to a water bath at 76°C for 1 hour for digestion of the plant material. The replica was washed, transferred to a 200-mesh grid, and examined with a Philips EM-200 electron microscope.

Figure 1 is a micrograph of a replica prepared in this way. The top edge shows the leaf surface with wax deposits and underlying cuticle, and the rest consists of the obliquely fractured cell wall. In the latter area one can observe cellulose fibrils, both singly and in groups, fractured along their length. The smaller cross section of the fibril ends distinguishes them from wax canals, and the latter can also be recognized if the wax attached to the replica is allowed to remain since this makes the canals electron-opaque.

Shadows of transverse fractures of the canals suggest that they have a central core usually 6 to 10 mμ wide and an overall diameter of approximately 40 mμ. These dimensions are similar to those observed in previous replica studies (*1*).

The lower left of Fig. 1 shows what appears to be a longitudinal fracture of a wax canal. Since the wax canal lies parallel to adjacent cellulose fibrils it is possible that its path may be influenced by them. A single wax pathway can seldom be traced along its full length from plasmalemma to cuticle since midway between, at perhaps

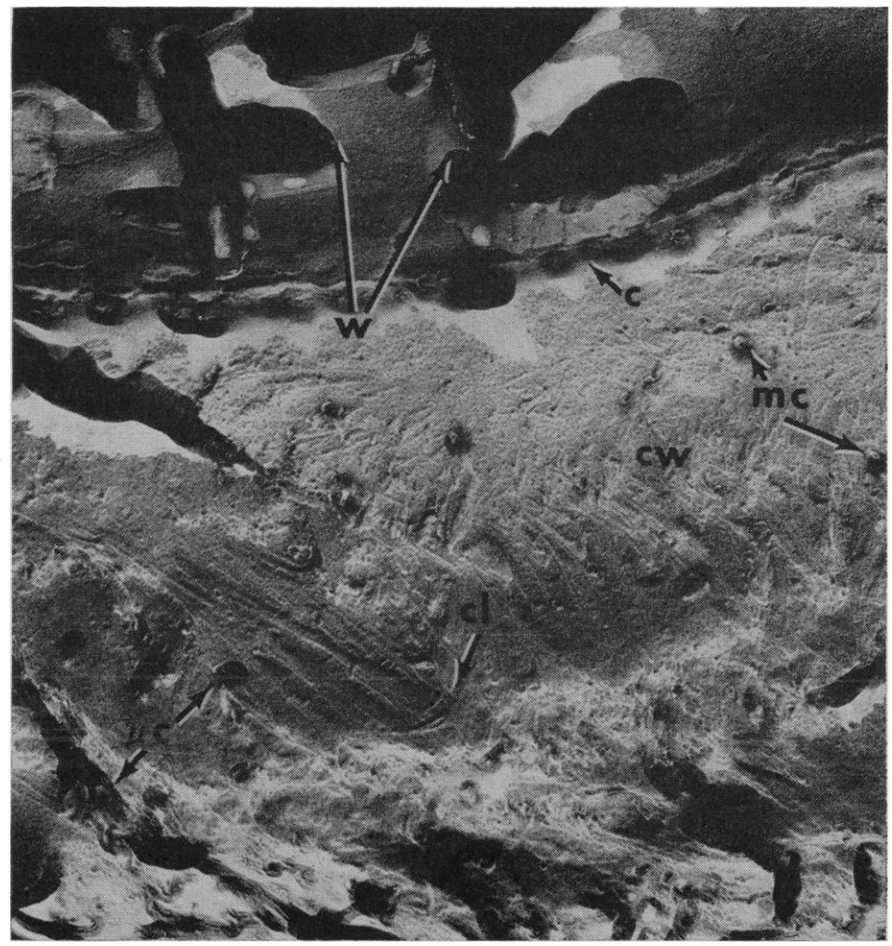

Fig. 1. Freeze-etch preparation of a white-clover leaf showing wax-covered adaxial surface and fractured cell wall containing cellulose fibrils and wax canals. Surface wax, *W*; cuticle, *C*; cell wall, *CW*; wax microchannels, *MC*; cellulose *CI* (× 75,000).

the junction of primary and secondary cell walls, there is generally an abrupt change of direction.

The wax canals observed in the freeze-etch replicas consist mainly of transverse cross sections or short longitudinal fractures. Since they are distributed throughout the cell wall and are often observed entering from the cuticle and plasmalemma, there is little doubt that they traverse its full width.

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Effect of Amantadine Hydrochloride on the Response of Human Lymphocytes to Phytohemagglutinin

Abstract. *Amantadine hydrochloride (Symmetrel), which is an antiviral agent marketed for the prevention of infection by influenza virus, inhibits the mitogenic response of human lymphocytes stimulated with phytohemagglutinin. The concentrations which effectively inhibited the response to phytohemagglutinin were similar to those which maximally inhibit virus replication. The drug inhibited the mitogenic response without affecting the ability of phytohemagglutinin to agglutinate leukocytes. The data suggest that phytohemagglutinin, amantadine, and certain lipid-containing RNA viruses take part in cell-membrane interactions of common nature.*

Phytohemagglutinin (PHA), a mucoprotein obtained from *Phaseolus vulgaris*, agglutinates erythrocytes and leukocytes and initiates a mitogenic response of lymphocytes. In the presence of PHA, 60 to 90 percent of the lymphocytes undergo blastic transformation, with the maximum response observed in 3 days. Exposure of lymphocytes to a specific antigen results in blastic transformation of up to 30 percent of the cells, and this response requires about 6 days. The response to PHA of lymphocytes from infants with congenital rubella was impaired during the early course of the disease (*1, 2*). In vitro, addition of rubella virus to lymphocytes from normal subjects similarly impairs the response of the cells to PHA (*2*) and specific antigens (*3*).

Amantadine (1-adamantanamine HCl) is one of the first antiviral drugs marketed for the prevention of viral diseases (Symmetrel). The antiviral activity of the drug is limited to certain lipid-containing viruses (*4–10*). Amantadine exerts an antiviral effect by preventing virus penetration into the cell and not by altering virus adsorption or other phases of virus replication (*7, 11*). During the attempt to determine whether penetration of rubella virus was necessary to inhibit the PHA response of lymphocytes, it was found that amantadine also inhibited the PHA response of lymphocytes. The inhibition of the mitogenic response of the lymphocytes was not associated with a loss of the leukoagglutinin effect caused by PHA.

Heparinized blood samples were obtained from normal adult donors; the erythrocytes were allowed to settle at 37°C, and the leukocyte-rich plasma was decanted. The leukocytes were sedimented by centrifugation, washed once in Hanks's balanced salt solution, and again suspended to a final concentration of 0.5 to 1×10^6 nucleated cells per milliliter in Eagle's medium containing 20 percent fetal bovine serum, antibiotics (100 units of penicillin and 100 μg of streptomycin per milliliter), and 1.5 g of bicarbonate per liter. Cultures containing 2 ml each were prepared in quadruplicate. Phytohemagglutinin-M (0.05 ml) (*12*) was added to appropriate cultures. Stock solutions of amantadine HCl (*12*) (1000 μg/ml), prepared in distilled water, were used fresh or were frozen at −20°C until used. Further necessary dilutions of the drug were made in the culture medium. All cultures were incubated for 3 or 5 days at 37°C before 0.06 or 0.2 μc of H^3-thymidine (specific activity, 2 c/mmole) was added to three culture tubes of each set. Five hours later, growth of the cultures was terminated by the addition of an excess of unlabeled thymidine, and the cells were separated by centrifugation. The cells were washed in cold Hanks's solution, cold 5 percent trichloroacetic acid, and cold methanol. The final precipitate was dissolved in 0.1 ml of Hyamine (*12*) mixed with scintillation fluid and was counted in a Beckman liquid-scintillation counter. Data represent the average number of counts per minute for three cultures of each set. The fourth culture was used to determine cell morphology and viability by the dye-exclusion method (*13*). The source, preparation, and assay of rubella virus have been described (*2*).

Amantadine effectively inhibits rubella virus replication (*5, 8, 9*); we performed experiments in which amantadine (final concentration, 50 μg/ml) was added to leukocyte cultures 30 minutes before the cultures received PHA with or without rubella virus (Table 1). There was a reduction of thymidine incorporation of 50 to 60 percent when rubella virus was added to the leukocyte cultures containing PHA. Amantadine alone, when added to the cultures, produced a 60- to 70-

Table 1. Effect of amantadine and rubella virus on the lymphocyte response to PHA (at concentration of 1:50). Leuko-agglutination was arbitrarily graded 0 to +2, with +2 being maximum agglutination.

PHA	Amantadine	Rubella virus	Count/min*	Transformation (%)	Leuko-agglutination	Viable (%)
			Trial 1			
			181	4	0	95
	+		371	5	0	97
		+	81	1	0	100
+			6,443	64	+2	91
+		+	3,375	40	+1	92
+	+		1,901	40	+2	93
+	+	+	2,116	30	+1	96
			Trial 2			
			121	5	0	93
	+		105	7	0	89
		+	151	3	0	90
+			14,736	86	+2	92
+		+	5,590	69	+2	90
+	+		5,606	51	+2	89
+	+	+	3,639	44	+2	94

*H^3-thymidine (0.06 μc) added 5 hours before termination of cultures.