STUDIES OF THE FINE STRUCTURE OF THE WAX LAYER OF SULTANA GRAPES

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Summary

The surface waxy bloom of sultana grapes has been studied using the carbon-replica technique in combination with electron microscopy. This layer consists of a series of overlapping wax platelets, each of which is about 0·1 μ wide. The air spaces between the wax platelets become filled with liquid when sultana berries are dipped in commercial emulsions known to accelerate their drying rate. Washing in distilled water removes this layer of dipping emulsion from the surface. The appearance of dipped and washed grapes is similar to that of untreated grapes.

Rubbing the grapes with lens tissue destroys the precise arrangement of wax platelets but does not remove the wax. Dipping the grapes in chloroform (four changes of 10 sec), a treatment known to remove most of the wax, leaves sufficient wax to enable replicas to be prepared. Treatment of the grapes with chloroform for longer periods and Soxhlet extraction removes all the waxy structures.

These results are discussed, and a hypothesis is presented which may explain the mechanism of action whereby dipping emulsions increase the rate of water movement from grapes.

I. INTRODUCTION

Despite the development of techniques for replicating and examining cuticular surface structures of plants at high resolution, relatively few plant species have been examined. Even fewer studies are available where an attempt has been made to correlate the presence of these waxy surfaces with the physiological properties of the organ.

An understanding of the structure and properties of waxy cuticules is of considerable importance to the dried fruit industry, where generally the aim is to bring about the rapid loss of water from fruits. In the districts in Australia where sultanas are grown commercially, climatic factors make it particularly desirable to accelerate the drying rates of grapes. The present practice with the most important drying grape, sultana (*Vitis vinifera* var. *sultana*), is to dip the grapes while still attached in bunches in an alkaline emulsion before drying. This dipping increases the rate of water loss about three-fold and produces a more readily marketable pale golden brown dried fruit. The proprietary dipping emulsions consist of varying mixtures of oleic acid, ethyl oleate, and sulphated butyl oleate, potassium carbonate, and water (Martin and Stott 1957).

Little is known of the mechanism of action of the dipping agents, despite a number of investigations of this problem (Dudman 1962; Dudman and Grncarevic 1962; Grncarevic 1963). The existing method was developed empirically and evolved from the Greek procedure of dipping grapes in emulsions of olive oil and potash before

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drying (see Grncarevic 1963). Previous workers have all emphasized the lack of information about the physical arrangement of the wax and about the possible effect of dipping agents upon this layer.

The present paper illustrates and describes the arrangement of the wax layer on the grape surface as resolved by means of carbon replicas and electron microscopy. It also describes the structure of the wax layers after they were subjected to a series of experimental treatments. Some of the treatments were designed to correspond to commercial methods known to accelerate drying, while others were laboratory methods used to remove wax from the berry. The results obtained are discussed and an explanation is offered for the role of wax in preventing the loss of water from biological systems such as the grape.

II. Methods

Grapes were grown at the Horticultural Research Section at Merbein, Vic., and selected bunches were air-freighted to Melbourne. Grapes used for microscopical study were carefully chosen so that the areas examined had not been subjected to any obvious physical damage.

(*a*) *Light Microscopy*

Light micrographs of the surfaces were made with a 4-mm objective and a prism vertical illuminator corrected for use without a coverglass. A filtered carbon arc light source was used and photomicrographs were made at an initial magnification of $\times 130$ on 35-mm film. A rapid photosensitive contraction of the grape whenever a low-power (tungsten filament) light source was temporarily removed from the specimen made it necessary to use a photomicrographic technique in which the grape was continually illuminated and in which the photographic exposure was a fraction of a second.

(*b*) *Electron Microscopy*

Tangential slices of grape were taken with a sharp razor blade. These included the epidermis and a small amount of fleshy tissue. This fleshy side was blotted to remove any free moisture and placed on a glass slide in an Edward's 12E6 vacuum-coating unit. A dish of fresh phosphorus pentoxide drying agent was placed near the specimen and sharpened carbon rod electrodes were about 14 cm above. A moderately thick layer of carbon was evaporated in a series of short bursts and this gave a pale grey-brown colour on a white porcelain plate. This was carried out in a vacuum of $2{\cdot}5 \times 10^{-4}$mm Hg attained within a pumping time of 12 min. The method followed was essentially that of Juniper and Bradley (1958) in which a 2% "Formvar" layer in ethylene dichloride was followed by a 5% "Bedacryl" layer (in benzene) and this backed with "Selotape". After stripping from the grape-skin surface the plastics were removed with acetone and chloroform and the resulting carbon replica was mounted on a 200-mesh copper grid. This was examined without shadowing in a Siemens Elmiskop I electron microscope at 80 kV. The resulting positive electron micrographs were photographed on Ilford special contrasty lantern plates, and from these 1 : 1 negatives were made on to Ilford fine grain ordinary N5·30 film from which the final prints were made.

(c) *Experimental Treatment of Grapes*

Individual grapes were dipped in commercial dipping emulsion (2% Voullaire's "Eemulsoyle" in 2·5% potassium carbonate) for 3 min with some agitation and allowed to drain on a wire rack in an air-conditioned room at about 65°F (18°C) and 65% R. H. Grapes dipped in chloroform were generally given four changes of 10 sec each. Some were given up to 15 min in one or two changes of chloroform. Soxhlet chloroform-extracted grapes were given a 2-hr treatment, which represents about 40 washes of the condensed chloroform. Washing of the grapes was achieved by holding them for about 20 sec in a jet of distilled water delivered from a wash-bottle.

III. Results

(a) *Cuticle Structure in Normal Untreated Grape Skin*

The waxy bloom (Plate 1, Fig. 1) on the surface of grapes (*V. vinifera* var. *sultana*), when seen as a carbon replica with the electron microscope, consists of a series of wax platelets overlapping one another and each, generally, is *c.* 0·1 μ wide (Plate 1, Fig. 3). The free edge of the platelet typically has a very undulant margin with a series of small and large lobes. At varying intervals some of these wax margins curve upwards to give what at first sight appears to be a second system of platelets lying with their edge vertical to the plane of the grape-skin surface (see arrow in Plate 1, Fig. 3). From the dimensions and scatter of these it seems likely that they are the features vaguely resolved as numerous small white flecks by means of vertically illuminated light microscopy of the grape surface (Plate 1, Fig. 2). The light micrographs do show one feature not revealed by our carbon-replica, electron-micrograph technique, namely the presence of numerous yeast colonies on the waxy surface. Hyaline structures interpreted as being these are indicated by arrow-heads in Plate 1, Figure 2. Some variation in structure as revealed by electron microscopy of carbon replicas was found particularly earlier in the season before the grapes were fully ripe (Plate 2, Figs. 1 and 2). In Plate 2, Figure 2, deep, radiating, semi-dichotomizing, narrow-lobed platelets of wax can be seen. The platelets in Plate 2, Figure 1, are somewhat intermediate in character between those found in the typical example in Plate 1, Figure 3, and the extreme example in Plate 2, Figure 2. The waxy bloom is markedly hydrophobic. A strong jet of water has little effect on the surface structure. It may result in a slightly greater number of wax platelets with their edges in a vertical plane to the surface of the grape, but it is difficult to be certain that this is a valid interpretation in view of the drastic treatment involved in the preparation of the specimens. Virtually all the water applied is shed. From this it is concluded that rain normally has little direct effect on the wax structure, and it seems certain that the so-called rain damage of grapes is not caused by the entry of water through the berry surface.

The arrangement of wax platelets is very easily disturbed by handling the grape. However, it is very difficult to remove the wax by physical polishing. The central grape in Plate 1, Figure 1, has had its lower half polished with a piece of lens tissue. Plate 4, Figure 4, shows the surface of such a grape as resolved by the light microscope where it can be seen that the surface is generally smeared but little wax is removed. A typical replica of such a "polished" surface is illustrated in Plate 4, Figure 3. The wax platelets appear to have coalesced into clumps.

(b) *Effect of Commercial Dipping Solutions*

Grapes were dipped for a 3-min period in commercial dipping emulsion and the surfaces were then replicated immediately on drying, i.e. about 1 min after dipping. The carbon was actually applied 10 min later, when vacuum conditions were established. Replicas were also prepared 1, 24, and 48 hr after dipping. Replicas were difficult to make on dipped material. Many had to be discarded due to the presence of clearly recognizable artefacts of the replica technique. Dipped grapes which were then washed with a jet of distilled water were easily replicated and the resultant replica was essentially similar to that of the undamaged waxy surface. Typical immediate replicas of a dipped grape and a dipped but washed grape are illustrated in Plate 3, Figures 1 and 2. The light micrographs of similarly treated grapes are illustrated in Plate 3, Figures 3–5. In Plate 3, Figures 3 and 4, the dipped fruit surface has become a "sea" of dipping emulsion and what may be interpreted as high areas of wax (see Plate 3, Fig. 1) are emergent above this sea. When grapes are washed with a jet of distilled water the light micrograph (Plate 3, Fig. 5) and the electron micrograph (Plate 3, Fig. 2) appear to be indistinguishable from that of the control (Plate 1, Figs. 2 and 3). A typical replica of a surface allowed to stand for 24 hr after dipping is illustrated in Plate 4, Figure 1. The dipping emulsion still forms a sea over the grape surface and this can still be removed by washing with water (Plate 4, Fig. 2). An essentially similar result was obtained with the 48-hr treatments, although by this time the grapes had shrivelled slightly and were showing signs of water loss.

(c) *Effect of Wax Solvents on Surface Wax*

From data of Martin (1960) and Dudman and Grncarevic (1962), three or four successive 10-sec dippings in chloroform have been widely accepted as an efficient method for removal of most of the wax from fruits being studied in relation to dried-fruit production. From a number of experiments and the present study it is concluded that while such a treatment removes a great deal of wax it still leaves sufficient surface wax to replicate recognizable waxy structures (Plate 5, Fig. 1). A longer period of immersion in chloroform, e.g. four changes of 30 sec each, causes a greater softening of remaining wax so that it tends to merge into a single corrugated layer (Plate 5, Fig. 2). The only really satisfactory method for removing almost all of the wax was a 2-hr Soxhlet extraction with either chloroform or petroleum ether. The resulting "cleaned" grape skin is difficult to replicate but small areas of carbon replica could be obtained (Plate 5, Fig. 3). These almost always had some associated "netting" which is almost certainly artefact. The light micrographs of the Soxhlet chloroform-extracted grape surface (Plate 5, Figs. 4 and 5) at least partially compare with the electron micrographs. The dark fissures may well be the cracks in the non-waxy cuticle remaining after solvent extraction. These have not been detected by the method of self-shadowed replication used.

IV. Discussion

Early work on the anatomy of grape berries by De Villiers (1926) indicates that the epidermis of grapes is generally similar to the epidermis of onion and other

higher plant leaves which have been described in detail by Orgell (1954, quoted by van Overbeek 1956) and Scott *et al.* (1958). These workers have shown that the epidermis of leaves consists of an outer layer of wax covering a cuticle, which is composed of a mixture of wax and cutin. Between the cuticle and the epidermal cells, which have cellulose-thickened walls, there is a layer of pectin.

The only avenues for water loss in grapes, which lack stomates (Eames and MacDaniels 1947), are via the pedicel or directly through the epidermis. Some water loss occurs through the pedicel in the process of grape drying, but the speed with which the pedicel shrivels indicates that this is not a major pathway. The main route of water loss is through the epidermis, and the work of Martin and Stott (1957), Dudman (1962), Dudman and Grncarevic (1962), and Grncarevic (1963) suggests that the wax layer may be the rate-limiting barrier. Within the epidermis there are probably successive and additive barriers to the transmission of water. The present results are primarily concerned with the role of the wax layer in the water economy of grapes.

The wax layer of sultana grapes, as revealed by the carbon-replica technique and electron microscopy, shows some similarities with the wax layers that occur on the leaf surfaces of the plant species investigated by Mueller, Carr, and Loomis (1954), Scott *et al.* (1958), and Juniper and Bradley (1958). The sultana wax consists of large flat platelets rather than rods which are a feature of many species.

Results obtained from both light and electron microscopy show that, on dipping, the commercial emulsion fills up the minute air spaces between the wax platelets. It appears that these emulsions have the capacity to convert the wax layer of sultanas, which is normally strongly hydrophobic, to a hydrophilic condition causing it to be "wetted". This effect can be observed macroscopically by the wet appearance of dipped grapes, a feature which persists for a considerable period (up to 24 hr) after dipping.

It is significant that both Dudman (1962) and Grncarevic (1963) have shown in washing experiments that treatment with dipping emulsion does not permanently alter the rate of water loss so long as the treated grapes are washed within 24 hr of drying. They also showed that commercial dipping emulsions do not exert their effect on grapes by removing significant amounts of surface wax. This led Dudman (1962) to suggest that emulsion dips might act by bringing about critical surface changes—presumably in the wax layer. The replicas prepared from grapes that had been dipped and then washed were similar to those prepared from untreated grapes, suggesting that the irreversible changes in wax structure which occur on dipping are small.

The other treatments which were applied in attempts to modify the wax layer of grapes were rubbing with lens tissue and washing with chloroform. The rubbing treatment did not remove the surface wax of sultanas but rather pushed it into heaps—a different effect from that reported for chrysanthemum by Juniper and Bradley (1958) who found that they could remove most of the wax from these leaves by stroking with a squirrel-hair brush. It was found in drying experiments that the rubbing treatment had little influence on rate of water loss. The wax appears to be smeared across the surface and in this state (Plate 4, Fig. 4) still acts as an efficient barrier to water movement.

Although chloroform treatment is claimed to remove the wax from plant cuticles (Martin 1960; Grncarevic and Dudman 1962), it was found in the present investigation that this treatment (four 10-sec changes) leaves sufficient wax on the grapes to form recognizable replicas. It should be stressed that replicas only record the surface appearance and provide little information about the actual quantity of wax on a particular surface. Treatment of grapes for much longer periods than 40 sec with chloroform by Soxhlet extraction completely removes the wax layer.

Grncarevic (1963) has recently provided comparative data for the effects of various treatments, including chloroform treatment on the drying rate of sultana grapes. He dried grapes under standardized conditions (50°C and a forced draught of 50 ft/min) and measured the time required for berries to reduce to 50% of their original weight. Untreated grapes took 45 hr to dry, while berries dipped in commercial emulsions or those treated with chloroform (four 10-sec changes) both took only 17 hr. Chloroform treatment of sultanas removes sufficient of the wax to eliminate this layer as a barrier for water loss. The remarkably similar drying rates of chloroform-treated and commercial-emulsion-dipped grapes suggests that commercial emulsions in some way also eliminate the resistance to water loss offered by the wax layer.

During drying of the normal grape, water diffuses as a liquid from the cells through the pectin and cuticular layers until it reaches the wax-platelet region. From here it is suggested it moves as a vapour through the sub-light-microscopic capillary spaces formed by the overlapping wax platelets. Water films or water droplets cannot form in this layer because of the hydrophobic nature of the wax. The length of the capillary pathway is not known but it must be many times that of the thickness of the wax layer which is estimated to be only a few microns in depth. In this system water vapour moves along a gradient created between the high suction pressure of the external atmosphere and the saturated conditions within the grape itself. Despite this gradient the effective length of the fine capillaries is such that the rate of water movement by vapour diffusion is slow.

In the dipped grape the emulsion forms a continuous aqueous zone permeating the capillary spaces between the now hydrophilic wax platelets. During drying, water leaves the cuticle layer in the liquid phase and enters the emulsion-filled capillaries. As water evaporates either from the wet surface of the grape or from the surfaces of the numerous irregular capillaries between the overlapping wax platelets, the forces of capillarity tend to drag more water from the grape. This process continues so long as the internal wax surfaces are maintained in the hydrophilic condition. In this way it is suggested that the dipping emulsion eliminates the slow process of vapour diffusion through capillaries and greatly accelerates the rate of water loss from grapes.

This hypothesis is consistent with both the light- and the electron-microscope observations. Further, it is supported by the results of Dudman and Grncarevic (1962), Dudman (1962), and Grncarevic (1963), who found that the effects of dipping emulsions are essentially reversible and that little wax is removed by dipping.

It is of interest here that preliminary trials have shown that rapid drying rates can also be achieved by dipping grapes in mixtures of commercial detergents (such as "Span 20") plus potassium carbonate. The addition of potassium carbonate seems to be necessary, and possibly acts by saponifying the fatty acids such as oleic, stearic,

and oleanolic acids which are known constituents of grape wax (Markley, Sando, and Hendricks 1938). The conversion of the wax from the hydrophobic to the hydrophilic condition is regarded as the important step, and it appears that potassium carbonate influences this reaction.

It must be stressed that this hypothesis only proposes to explain how dipping solutions overcome the resistance to water loss offered by the wax layer of the grape epidermis. The mechanism proposed for the control of water loss in untreated grapes may also apply to the movement of water through the waxy-covered, stomate-free, epidermal surfaces of leaves.

V. Acknowledgments

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VI. References

De Villiers, F. J. (1926).—Sci. Bull. Dep. Agric. S.Afr. No. 45.

Dudman, W. F. (1962).—*Aust. J. Sci.* **25**: 168–9.

Dudman, W. F., and Grncarevic, M. (1962).—*J. Sci. Food Agric.* **13**: 221–4.

Eames, A. J., and MacDaniels, L. H. (1947).—"An Introduction to Plant Anatomy." (McGraw-Hill Book Co.: New York.)

Grncarevic, M. (1963).—*Amer. J. Vit.* (In press.)

Juniper, B. E., and Bradley, D. E. (1958).—*J. Ultrastruct. Res.* **2**: 16–27.

Markley, K. S., Sando, C. E., and Hendricks, S. B. (1938).—*J. Biol. Chem.* 123: 641–9.

Martin, J. T. (1960).—*J. Sci. Food. Agric.* **11**: 635–40.

Martin, R. J. L., and Stott, G. L. (1957).—*Aust. J. Agric. Res.* **8**: 444–59.

Mueller, L. E., Carr, P. H., and Loomis, W. E. (1954).—*Amer. J. Bot.* **41**: 593–600.

Orgell, W. H. (1954).—Ph. D. Thesis, University of California.

Overbeek, J. van (1956).—*Annu. Rev. Pl. Physiol.* **7**: 355–72.

Scott, F. M., Hamner, K. C., Baker, E. and Bowler, E. (1958).—*Amer. J. Bot.* **45**: 449–61.

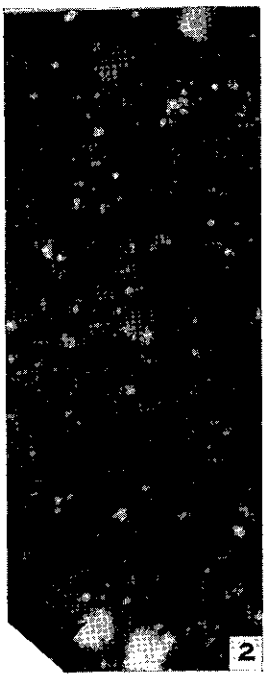
Explanation of Plates 1–5

Reference numbers are also given of plates stored at the Electron Microscope Unit, Botany School, University of Melbourne

Plate 1

Fig. 1.—Single grapes of *Vitis vinifera* var. *sultana*. *Left*, a grape with its waxy bloom undamaged. *Centre*, a grape with its lower half polished in an attempt to remove wax. The waxy bloom on the upper half is still mostly intact. *Right*, a grape which has been immersed in commercial dipping emulsion and allowed to drain for several hours. Surface of fruit after this treatment has a sheen which superficially resembles a polished grape.

WAX LAYER OF SULTANA GRAPES



WAX LAYER OF SULTANA GRAPES

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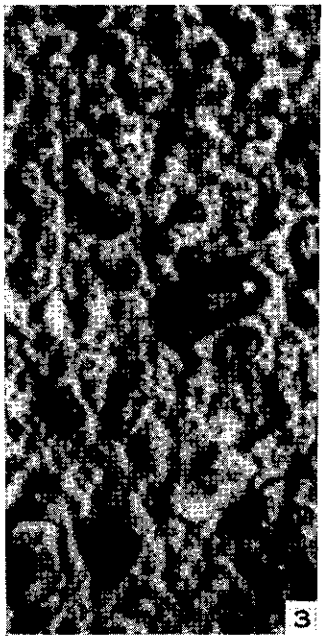

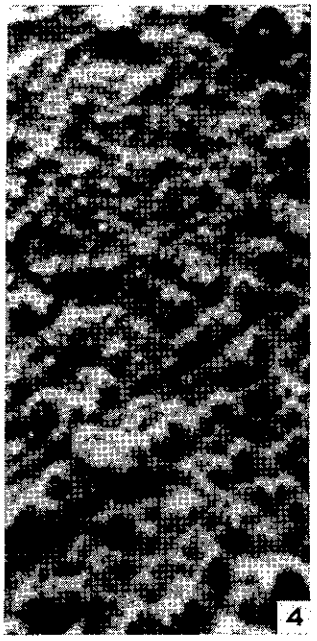

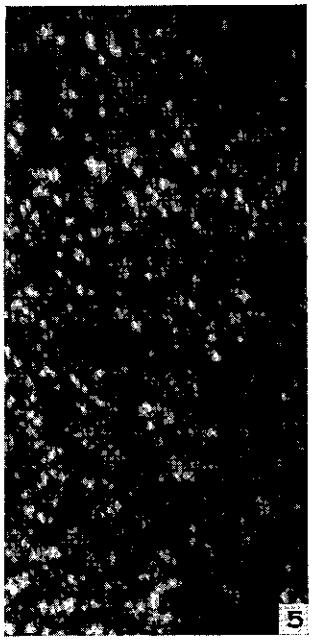

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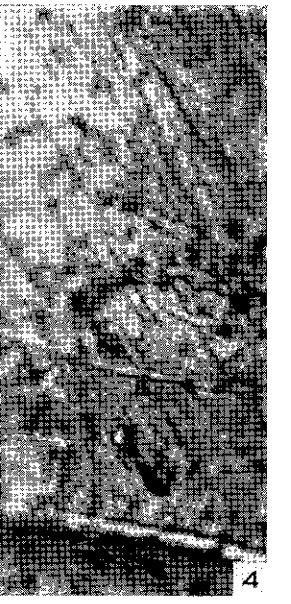

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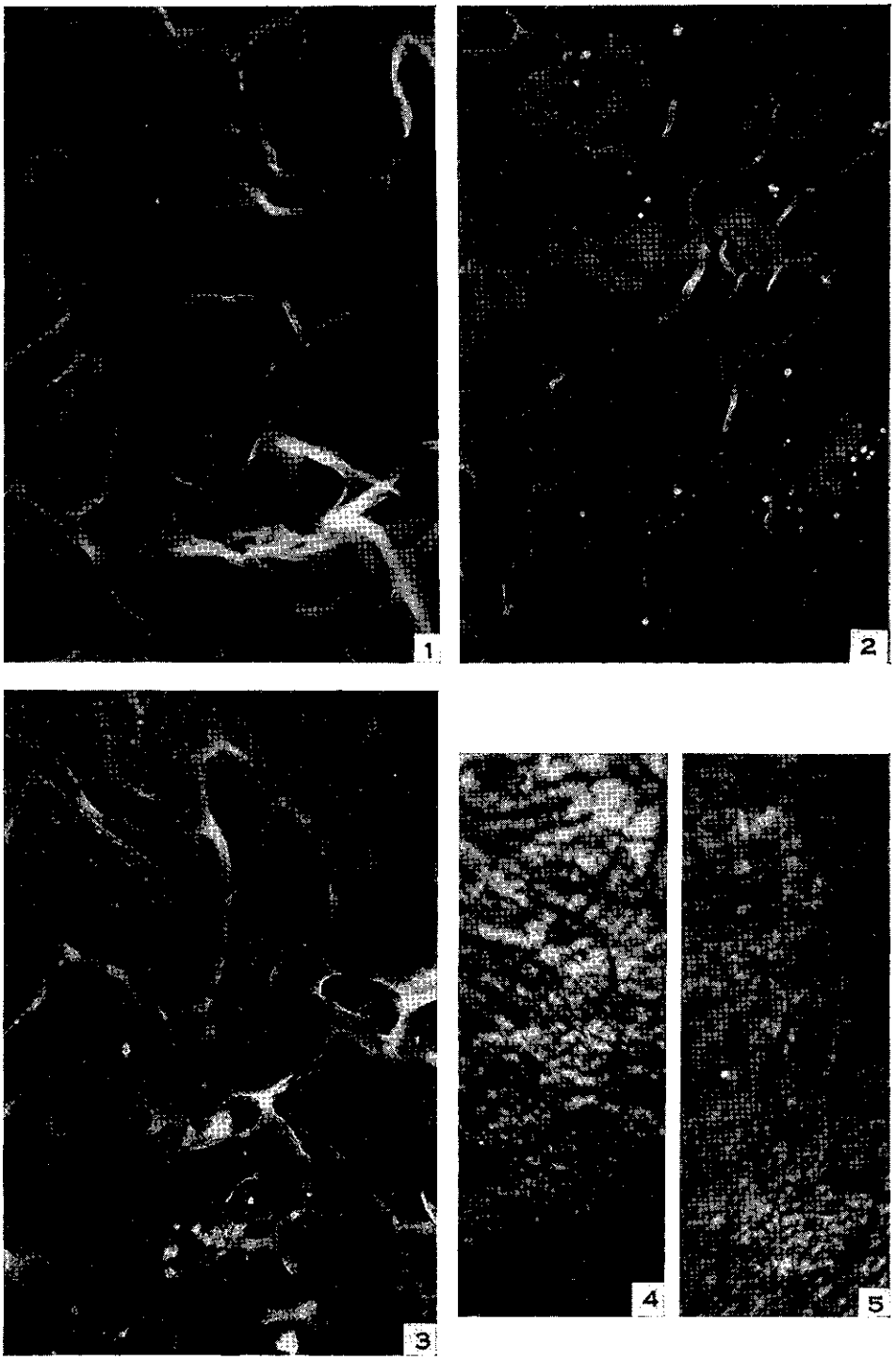

Fig. 2.—Vertically illuminated light micrograph of undamaged grape surface. ×530.

Fig. 3.—Typical arrangement of wax platelets as shown by an electron micrograph of a carbon replica of undamaged grape surface. No. 1101. 3·5×5000 = ×16,000.

PLATE 2

Typical variations seen in the waxy cuticle of *V.vinifera* var. *sultana* grapes as shown by electron micrographs of carbon replicas. Both types of wax illustrated were seen in slightly immature material early in the season

Fig. 1.—Deeply lobed wax platelets tending to dichotomize as they extend. No. 1100. 3·5×10,000 = × 35,000.

Fig. 2.—Very deeply lobed wax platelets with finger-like processes radiating from scattered centres on the surface. No. 1292. 3·5×5,000 = ×17,500.

PLATE 3

Fig. 1.—Carbon replica of grape surface immediately after immersion for 3 min in commercial dipping emulsion. No. 1641. 2×5,000 = ×10,000.

Fig. 2.—As for Plate 3, Figure 1, but washed following dipping. No. 1646. 2×5,000 = ×10,000.

Figs. 3 and 4.—Vertically illuminated light micrographs of grape surface following immersion for 3 min in commercial dipping emulsion. ×530.

Fig. 5.—As for Plate 3, Figures 3 and 4, but washed before taking photomicrographs. ×530.

PLATE 4

Fig. 1.—Carbon replica of grape surface made 24 hr after a 3-min immersion in commercial dipping emulsion. No. 1827. 2×5,000 = ×10,000.

Fig. 2.—Same treatment as for Plate 4, Fig. 1, but washed just before replica made. Even after coating of waxy layer with dipping emulsion for 24 hr, a brief wash with distilled water has made it possible to obtain a replica essentially similar to that of the control. No. 1672. 2×5,000 = ×10,000.

Fig. 3.—Carbon replica of fruit surface which had been polished with a piece of lens tissue. Apparently this has removed very little wax but has caused it to be rubbed into large amorphous masses. No. 1309. 2×2,000 = ×4,000.

Fig. 4.—Light micrograph of grape surface after identical treatment to that given for Plate 4, Figure 3. ×530.

PLATE 5

Fig. 1.—Carbon replica of grape after the "standard" treatment for removal of wax, i.e. four changes of 10 sec each in chloroform. No. 1650. 1·5×8,000 = ×12,000.

Fig. 2.—Carbon replica of grape surface after immersion for 15 min in chloroform at room temperature. No. 1337. 1·5×8,000 = ×12,000.

Fig. 3.—Carbon replica of grape surface after Soxhlet extraction in chloroform for 2 hr. No. 1652. 2×5,000 = ×10,000.

Figs. 4 and 5.—Light micrographs of grape surfaces after similar treatment to Plate 5, Figure 3.