INFRA-SPECIFIC VARIATION IN WAX ON LEAF SURFACES

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Summary

The structure of the surface wax on green and glaucous variants in *Eucalyptus urnigera*, *Poa colensoi* (natural cline forms), *Pisum sativum*, and *Brassica oleracea* (mutants) are described as seen under the electron microscope. Preliminary chemical data are also given. The green forms always possess wax deposits which are either smooth films on the cuticle or consist predominantly of platelets which lie flat on the cuticular surface. In some cases the platelets tend to be arranged in parallel groups. In the glaucous variants, the wax consists predominantly of rods or filaments growing outwards from the leaf surface and presenting many light-scattering surfaces.

Chemical data show a higher β-diketone content with wax from glaucous plants in both *Eucalyptus* and *Poa*. Glaucous *Brassica* has a higher ketone content.

I. Introduction

In many species of plant, genes controlling the structure of the leaf surface have been distinguished. Most of these alter the light-reflecting properties of the leaf, changing a glaucous leaf with a waxy "bloom" to a full green without "bloom". Several workers (Mueller, Carr, and Loomis 1954; Schieferstein and Loomis 1956; Juniper 1959) have shown by electron microscopy that the glaucous appearance of many leaves is due to the presence of light-scattering deposits of wax on the surface of the cuticle. Juniper has followed the development of the wax deposits when etiolated leaves, which are non-glaucous, are exposed to light.

This paper gives a preliminary account of a study under the electron microscope of recessive mutants in peas and cauliflowers in which the normal waxy glaucousness appears reduced in amount or quite absent.

In addition to genetical variation which has arisen by recent mutation in cultivated plants, many species show clinal or ecotypic variation in leaf colour in wild populations. Examples include several species of eucalypt (Barber 1955; Barber and Jackson 1957), grasses (e.g. Daly 1961). Transplant experiments have shown that the differences between these clinal forms are also genetically controlled, although several genes are probably involved.

The genes controlling these differences could act in several different ways. They could suppress wax formation to a greater or less degree; they could alter the physical conditions under which crystallization of the exuded wax takes place; or they could alter the chemical composition of the wax, which in turn could alter the crystalline form.

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We shall see that the quantity of wax may be altered by gene action. However, preliminary chemical analyses have shown that in most, if not all, cases studied chemical differences are also present.

II. Materials and Methods

(a) *Electron Microscopical Investigations*

(i) *Plant Material*.—A cabinet controlled at 23°C and 80% R.H. was used to raise plants from seed of the following:

(1) *Eucalyptus urnigera*: the seed came from trees growing at 2000, 2300, and 3200 ft on Mt. Wellington, Tas. (For a detailed description of the cline, see Barber and Jackson 1957). Trees from 2000 ft are non-glaucous (G_0 in their terminology); from 2300 ft, mixed G_0 and G_1 (the weakest grade of glaucousness); from 3200 ft, G_3, the maximum and persistent grade.

(2) *Brassica oleracea* (cauliflower) F_2 cv. Blightproof × Paleleaf: both varieties are commercial varieties available in Australia. Blightproof has green wettable leaves, whilst Paleleaf has the typical water-repellent, waxy glaucous leaves of *B. oleracea*. The difference is inherited as a simple Mendelian difference, green being recessive to glaucous (Barber, unpublished data). It is not clear whether this mutant is identical with any already described in the literature, e.g. by Anstey and Moore (1954), or Thompson (1963).

(3) *Pisum sativum* (peas): normal glaucous and three genetically different mutants were examined in detail. They are:

Normal glaucous:	cv. Kelvedon Monarch, obtained from commercial sources in New Zealand.
Subglaucous:	Line 6 obtained from Lamm. This line is recessive for the mutant *wa*, which reduces glaucousness very considerably.
Subglaucous:	Line 25 of Lamm, recessive for mutant *wb* which phenotypically is similar to *wa*.
Green:	Line 31 of Lamm, recessive for *wb* and *wlo*.

Descriptions of the phenotypes of the mutants and their linkage relationships are given in Lamprecht (1961).

(4) *Poa colensoi*: This was obtained from the South Island of New Zealand and examined before deterioration. The glaucous plants were typical of plants from the low rainfall districts of Alexandra and Lake Pukaki (15–20 in. per annum); the non-glaucous were typical of the high rainfall districts of Craigieburn and East Sabine River (70 in. per annum). Daly (1961) gives a detailed description of the variations and their ecology.

(ii) *Electron Microscopy*.—Carbon replicas of the leaf surfaces, preshadowed with gold–palladium, were prepared by methods described by Hall and Donaldson (1963). They were examined with a Philips EM100B electron microscope operated at 60 and 80 kV.

(iii) *Contact Angles*.—The wettability of each leaf surface was obtained from measurements of the contact angles which a 2 mm water droplet made with the leaf surface in the manner described by Fogg (1947). A microprojector with a magnification of $\times 50$ was used to project the profile of the droplet on to a ground-glass screen.

(*b*) *Chemical Investigations*

(i) *Plant Material*.—Plants were grown in the garden at Hobart and flown to Melbourne packed in plastic bags. The two samples of *E. urnigera* were collected from two saplings 8 years old grown at the Collinsvale Farm, University of Tasmania, from seed collected at 2400 ft (green G_0) and 3200 ft (glaucous G_3).

(ii) *Chemical Methods*.—The wax was extracted by dipping fresh plant material into a beaker containing boiling light petroleum (free from aromatic hydrocarbons and boiling range 60–80°C). Infrared absorption spectra of wax samples were compared, approximately 2% solutions in carbon tetrachloride being used. Details of the methods of analysing *Eucalyptus* waxes have already been described (Horn, Kranz, and Lamberton 1964).

III. Results

(*a*) *Electron Microscopy*

(i) *Eucalyptus urnigera*

Two forms of wax are present on the leaves of *E. urnigera*. One form consists of long round rodlets ($0 \cdot 2$–$0 \cdot 3$ μ diameter) that branch at irregular intervals as they progress outwards from the leaf. Where this form of wax is dense the rodlets make an interlocking mesh with the outer surface as much as $2 \cdot 5$ μ from the cuticle. If the pseudo-replicas are immersed in a wax solvent long enough to dissolve the rodlets, the thin carbon film surrounding them sometimes collapses and the rodlets then appear to be ribbons.

The second form of wax consists of flakes which mostly lie flat on the leaf surface and have a thickness of about $0 \cdot 1$ μ. The surface structure of the flakes is shown in Plate 1, Figure 1, inset. Some of the flakes lie at an angle to the leaf surface, with the serrated edge furthest from the leaf.

The flaky form of wax predominates on leaves of the 2000 ft type and while a few rodlets are also present on the adaxial surface, as in Plate 1, Figure 1, they are more plentiful on the undersurface of the leaf.

At 3200 ft the leaves of juvenile plants are covered with a thick growth of the rodlets (of the form shown in Plate 1, Fig. 2, inset) in addition to the flakes. When the rodlets are plentiful, as in Plate 1, Figure 2, however, their shadows hide the underlying flakes. Replicas of leaves from 2300 ft type plants had an appearance intermediate between those of the 3200 ft and 2000 ft types. A mixture of rodlets and flakes are present, with the rodlet type wax being more plentiful on the abaxial surface.

(ii) *Poa colensoi*

The submicroscopic wax deposits on the leaves of plants from the low and high rainfall areas differed markedly. Plate 2, Figure 1, shows the deposits on a leaf from a low rainfall area and it can be seen that the wax consists of numerous rodlets of about $0 \cdot 3$ μ diameter and 2 μ long. The rodlets, which often appear to be tubular, are of fairly uniform length and there is little evidence of branching. Each rodlet appears to be exuded separately at the leaf surface and does not have a common base with others.

Wax typical of plants from high rainfall areas is shown in Plate 2, Figure 2. It consists of very thin strands (width approx. $0 \cdot 04$ μ) that lie flat on the leaf surface and are connected to areas of flat perforated sheet of the same thickness. Some small platelets are also present.

(iii) *Brassica oleracea*

On the adaxial surface of glaucous leaves, the wax crystals form mainly herring-bone patterns with as many as 20 rodlets connected near their middles, as in Plate 3, Figure 1. Many are asymmetrical, however, and appear to have dendritic crystallization patterns such as those formed by frost. The rodlets are about $0 \cdot 1$ μ in diameter and are up to 8 μ long. Each rodlet has minor projections along its length at spacings of approximately $0 \cdot 15$ μ.

The non-glaucous mutant has smooth mounds of wax about 1 μ across and the quantity of surface wax appears much less than on the glaucous plants. Plate 3, Figure 2, shows a typical non-glaucous surface that includes a few small deposits of the type present on the glaucous *Brassica*. The yields of wax as percentage fresh weight for the two types were: glaucous: $0 \cdot 033$–$0 \cdot 042$; green: $0 \cdot 016$.

Dr. F. Radler has estimated the amount of wax per square centimetre of lamina on the two types of leaf. A standard dipping time in solvents was used for these estimations and the following results were obtained: normal glaucous: 87 μg; green mutant: 52 μg. These results must be treated with caution as they represent only the wax dissolved in a short period from the surface and are not the total surface deposits (Hall, unpublished data). The values indicate, however, that the green has about 60% of the readily soluble wax present on the glaucous. Plate 3, Figures 1 and 2, give the impression that the green has much less wax than this, but when replicas of the green surface were examined at higher magnifications flat crystalline deposits of wax such as are shown in Plate 3, Figure 2, inset, appeared to cover the cuticle surface.

(iv) *Pisum sativum*

Wax on the glaucous normal differs in both form and distribution compared with that on the three mutants. On both surfaces of the leaf of Kelvedon Monarch the deposits consist of both rods and irregularly shaped platelets distributed in a random fashion, as in Plate 4, Figure 1. On the adaxial surface of the leaf the sub-glaucous mutant *wa* has wax consisting of a mixture of very small flat platelets and others which are larger than those on Kelvedon Monarch. In many areas, the platelets are arranged in concentric circles as in Plate 4, Figure 2, possibly round the centre of epidermal cells. This difference in orientation is more pronounced on the abaxial surface of the leaf where the wax consists of rodlets arranged in groups of about 20.

The structure of the wax on the adaxial surface of the other lines is shown in Plate 4, Figures 3 and 4. In *wb* the wax consists entirely of large platelets whilst in the double mutant *wb wlo* the wax is reduced to small spherical granules and a few rods, platelets being absent.

(*b*) *Wettability Measurements*

Contact angles of water droplets on the eucalypts and brassicas are recorded in the following tabulation. Each result is the mean of 40 measurements and standard deviations are also given.

Species	Adaxial Surface	Abaxial Surface
E. urnigera		
2000 ft	$99 \cdot 8° \pm 0 \cdot 9°$	$108 \cdot 6° \pm 1 \cdot 0°$
2300 ft	$113 \cdot 6° \pm 0 \cdot 9°$	$114 \cdot 5° \pm 1 \cdot 6°$
3200 ft	$141 \cdot 3° \pm 1 \cdot 1°$	$143 \cdot 0° \pm 0 \cdot 4°$
Brassica		
Non-glaucous	$109 \cdot 9° \pm 2 \cdot 5°$	$111 \cdot 3° \pm 2 \cdot 7°$
Glaucous	$>150°$	$>150°$

(*c*) *Chemical Composition*

Preliminary chemical data show that the physical differences between green and glaucous forms are accompanied by differences in the chemical composition of the waxes from *Poa*, cauliflower, and *E. urnigera*. In the case of peas the differences between the wax samples are not so well defined, and a more detailed study of pea waxes is continuing.

The results on cauliflower, eucalypt, and *Poa* wax are given in Table 1. The major difference between the cauliflower samples is the variation in the proportion of ketones, with a corresponding change in the amount of the combined alcohol and ester fraction. This difference was indicated at once by the infrared spectra of the waxes. The wax from glaucous cauliflower has a strong band at 1705 cm^{-1} (ketone) with a relatively weak shoulder at 1725 cm^{-1} (ester). In the spectrum of the wax from green cauliflower the intensities of the two carbonyl bands are reversed, the ester band being relatively strong, and the ketone band at 1705 cm^{-1} reduced to a weak shoulder. This observation is supported by gas chromatographic analysis of the wax. The whole wax was chromatographed so as to obtain an accurate measure of the relative proportion of hydrocarbons (principally n-nonacosane) and ketones (principally n-C_{29} ketone, probably n-nonacosan-15-one as in cabbage wax). Pure n-nonacosane and n-nonacosan-15-one from cabbage wax were used as reference compounds (Horn, Kranz, and Lamberton 1964). The results (Table 1) show a low ketone content in the green variety, relative to the amount of hydrocarbons. The wax from the dominant glaucous variety contains about three times as much ketone as the wax from the recessive green. It would be interesting to investigate whether the different proportion of ketone is balanced by an increased amount of secondary alcohols in the second instance, as these could arise by reduction of the ketone.

Purdy and Truter (1963) have recently reported a full analysis of the wax from glaucous cabbage (Winnigstadt). Their sample of wax contains intermediate amounts

TABLE 1

COMPOSITION OF WAXES FROM GLAUCOUS AND GREEN PLANTS

Species	Fraction of Wax	Amount in Glaucous Plants (%)	Amount in Green Plants (%)	Comments
Brassica oleracea	Hydrocarbons	31	30	Consisting largely of n-nonacosane
	Ketones	24	8–9	Largely a single ketone coinciding in retention time with n-nonacosanone
	Esters, alcohols, etc.	45	61–62	—
Eucalyptus urnigera	β-Diketones	52	7–8	Almost pure n-tritriacontan-16,18-dione, n-$C_{15}H_{31}COCH_2COC_{15}H_{31}$
	Hydrocarbons	2·7	25–26	Principal hydrocarbon n-$C_{29}H_{60}$ in glaucous variety and n-$C_{27}H_{56}$ in green variety
	Esters	10–12	10–21	Esters from green variety show a band at 1694 cm^{-1} (CCl_4) not present in the infrared spectrum of esters from wax of glaucous variety
	Triterpene γ-lactone acetate	*c.* 1	*c.* 3	11,12-dehydroursolic lactone acetate (Horn and Lamberton 1964)
	Flavones	*c.* 1	*c.* 3–4	Approximately equal parts of 5-hydroxy-7,4′-dimethoxy-6,8-dimethylflavone and 5-hydroxy-7,4′-dimethoxy-6-methylflavone (Lamberton 1964)
*Poa colensoi** (4 specimens–A, B, C, and D)		A B C	D	
	β-Diketones†	48 25 21	0	
	Hydrocarbons	10 15 18	35	
	Esters (unhydroxylated)	11 12 15	19	

* The remainder of the *P. colensoi* wax is composed of free acids, free alcohols, and other hydroxy compounds.

† $E^{1\%}_{1\,cm}$ in hexane at $\lambda_{max.}$ 273 mμ is proportional to the β-diketone content of the wax, and for specimens A, B, C, and D equalled 136, 95, 93, and 0, respectively.

of ketones (about 14%) and slightly more hydrocarbons (36%). Horn, Kranz, and Lamberton (1964) examined the wax from a glaucous cabbage of undetermined variety and found hydrocarbons (35%) and ketones (25%). Unlike Purdy and Truter, who found the ketone fraction to consist of a mixture of nonacosan-15-one (70%) and nonacosan-10-one (30%), the ketone fraction in this case consists of nonacosan-15-one with only trace amounts of other ketones which do not include nonacosan-10-one. This supports the view that there is considerable varietal difference in the composition of the ketone fraction. Purdy and Truter suggest that the ketones serve as precursors for the hydrocarbons by way of the corresponding alcohols. If this is the case the dominant gene must be acting as an inhibitor of these reduction processes.

TABLE 2

EUCALYPTUS URNIGERA HYDROCARBONS (COMPOSITION, WEIGHT %)

Carbon Number	Hydrocarbon Content		Carbon Number	Hydrocarbon Content	
	Glaucous Plant	Green Plant		Glaucous Plant	Green Plant
21	2·7%	—	26	4·7%	2·6%
22	2·4	—	C	1·3	—
23	5·7	1·2%	27	14·2	44·2
24	4·2	1·2	D	7·6	—
A*	2·4	—	28	12·5	4·0
25	11·8	21·4	29	26·6	24·0
B	3·9	—	31	—	1·4

* Wax hydrocarbons from glaucous leaves contain four minor components (A, B, C, D) which are not straight-chain hydrocarbons.

The situation in *E. urnigera* is clearly more complex. Similar wax yields were obtained from the two varieties (glaucous 0·36; green 0·30% of fresh weight), but as shown in Table 1 there is very much more β-diketone, consisting of almost pure n-tritriacontan-16,18-dione, $CH_3(CH_2)_{14}COCH_2CO(CH_2)_{14}CH_3$, in the wax from the glaucous variety than in the wax from the green variety. On the other hand the wax from the green variety has a much higher hydrocarbon content. The hydrocarbons from the two varieties differ not only in yield but also in the chain length distribution of the individual hydrocarbons present (Table 2). Gas chromatographic analysis shows that in the hydrocarbons of the glaucous variety n-$C_{29}H_{60}$ is the principal hydrocarbon, but in the green variety n-$C_{27}H_{56}$ predominates. Even these differences are difficult to explain on the basis of a single gene, but when the differences in other components are considered also, it appears, as progeny analysis already indicates (Barber, Crowden, and Jackson, unpublished data), that several genes are active in determining the differences in leaf surface in this species.

Estimation of some of the major constituents of the waxes from four plants of *Poa colensoi* (Table 1) has given similar results. Of the four plant specimens examined three were classified as glaucous and the waxes from these all contained β-diketones in amounts ranging from a maximum of 48% down to 21%. Full analysis of the

β-diketones has not been made, but $C_{19}H_{39}COCH_2COC_9H_{19}$ was a major component in one case. The wax from the non-glaucous plant of *P. colensoi* contained no β-diketones. An inverse relationship applies to the hydrocarbon content of the *Poa* waxes. As with *E. urnigera*, the wax from the non-glaucous plant contained the most hydrocarbons (35% of the total wax), with lesser amounts (10–18%) in the waxes from the glaucous plants.

In preliminary experiments made with pea waxes only small amounts of wax were available and positive identification of components has not proceeded far. The yield of wax from the four types was approximately the same, being about 0·04% of the fresh weight. All pea wax samples had essentially the same infrared absorption spectra, and these show that the waxes consist of esters, alcohols, and small amounts of acids and possibly ketones. It has also been shown that hydrocarbons are present. Gas chromatography of the whole wax showed some variation in the ratio of components present, but major chemical differences such as those found with *Poa*, eucalypt, and cauliflower have not been revealed. A more detailed study of pea waxes is being undertaken to define the extent of the chemical differences.

IV. Discussion

The electron microscope studies have shown that the glaucous appearance of leaves is associated with:

(1) wax deposits that grow outwards from the leaf surface;

(2) random orientation of the wax; and

(3) a leaf surface that is well covered with exuded deposits.

Subglaucous or non-glaucous surfaces on the other hand have wax deposits that:

(1) are mainly of a form which lies flat on the cuticle, i.e. the top surface of each deposit is almost parallel to the cuticle surface;

(2) are sometimes less plentiful than on a glaucous surface; or

(3) are orientated in a well-defined manner.

The surface wax on glaucous leaves is better equipped to scatter more light than the deposits on non-glaucous, mainly because their submicroscopic form and distribution produce more light-scattering surfaces per unit area. Plants may possess heavy deposits on their leaves or fruit but if this lies flat on the cuticle and has little structure, as on apples (Hall, unpublished data), and hence few light-scattering surfaces, then the cuticle has a shiny appearance and may be called waxy but not glaucous.

We have found that when contact angle measurements are combined with visual observations of glaucousness they give a good indication of the type of wax structure on the leaf surface. Leaves that are glaucous and produce contact angles greater than 120° have many deposits of rodlet or platelet type of wax which stands out from the leaf surface. Contact angles exceeding 145° have only been obtained when numerous wax rodlets or platelets cover a glaucous leaf surface, making it impossible for water droplets to come in contact with the cuticle; Plate 1, Figure 2, inset, Plate 2, Figure 1, and Plate 3, Figure 1, are examples of this.

Values lower than 110° indicate that the droplet is in contact with more of the hydrophilic cuticle surface. This is reflected in measurements of *E. urnigera*. The rodlet type of wax is predominant at 3200 ft and prevents water droplets from making contact with the cuticle. At lower altitudes this type of wax becomes progressively less and the contact angle decreases from 143° to 100°.

It is interesting that in both the eucalypt and *Poa* high-reflecting rodlet wax is associated with the high β-diketone content and with higher ketone content in *Brassica*. Whether this correlation indicates a significant relationship between the shape of the surface wax deposits and chemical structure can be determined only by further work. The waxes from many highly glaucous species of eucalypts have been shown to have a high β-diketone content (Horn, Kranz, and Lamberton 1964). The white powdery material rubbed from very glaucous juvenile leaves and stems of *E. globulus* by gently brushing with cotton-wool contains approximately 74% long-chain β-diketones, and the wax extracted from very glaucous leaf tips of *E. globulus* had a β-diketone content which was almost as high (70%). Somewhat less-glaucous leaves from further back along the same branch gave a wax containing 50% β-diketones. Among the eucalypts high β-diketone content is not essential for leaf glaucousness, for example *E. intertexta* is highly glaucous but the wax contains no β-diketones.

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Explanation of Plates 1–4

Plate 1

Fig. 1.—Mosaic showing predominance of flakes on adaxial surface of leaf of *Eucalyptus urnigera* from population growing at 2000 ft. ×2000. *Inset*: Wax flakes of *E. urnigera* lying flat on the cuticle surface. ×11,900.

Fig. 2.—Mosaic showing distribution of rodlets on adaxial surface of leaves of *E. urnigera* from population growing at 3200 ft. ×1750. *Inset*: Wax rodlets that form a mesh above cuticle of *E. urnigera*. ×8400.

Plate 2

Fig. 1.—Glaucous leaf surface of *Poa colensoi*. ×4600.

Fig. 2.—Non-glaucous leaf surface of *P. colensoi*. ×4600.

Plate 3

Fig. 1.—Wax deposits on leaf surface of glaucous *Brassica*. One stoma is visible. ×1850.

Fig. 2.—Surface of non-glaucous *Brassica* mutant. One stoma is visible. ×1850. *Inset*: Flat crystalline wax covering the leaf surface of non-glaucous *Brassica* mutant. ×33,000.

Plate 4

Adaxial leaf surface of normal glaucous and three mutants of *Pisum*. ×3200.

Fig. 1.—Normal glaucous (Kelvedon Monarch): genotype *wa, wb, wlo*.

Fig. 2.—Subglaucous: *wa, wb, wlo* (line 6).

Fig. 3.—Subglaucous: *wa, wb, wlo* (line 25).

Fig. 4.—Green: *wa, wb, wlo* (line 31).

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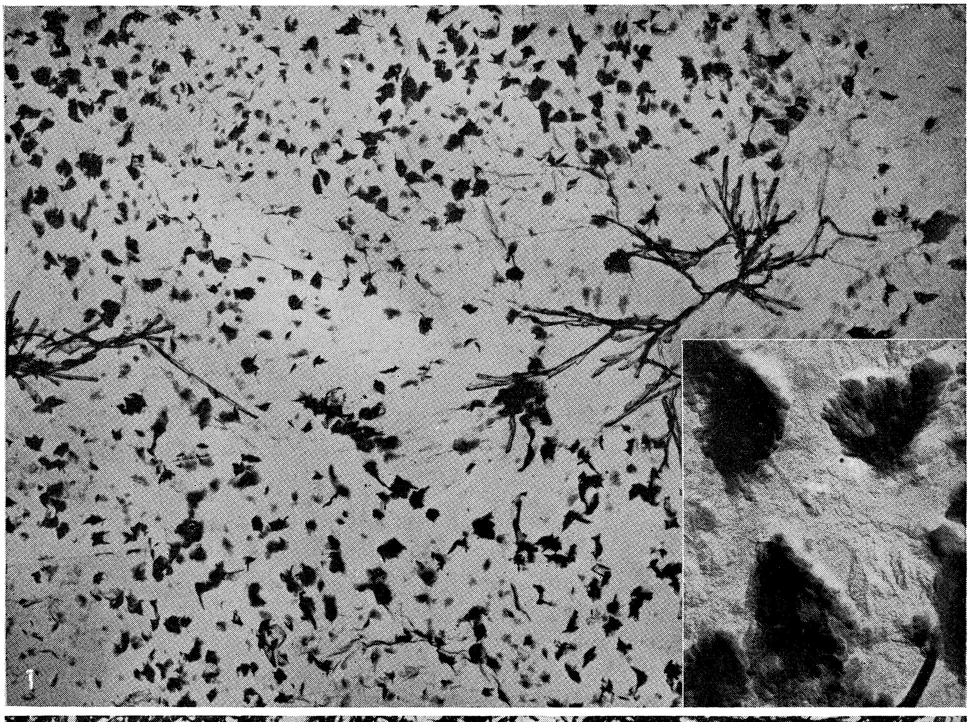

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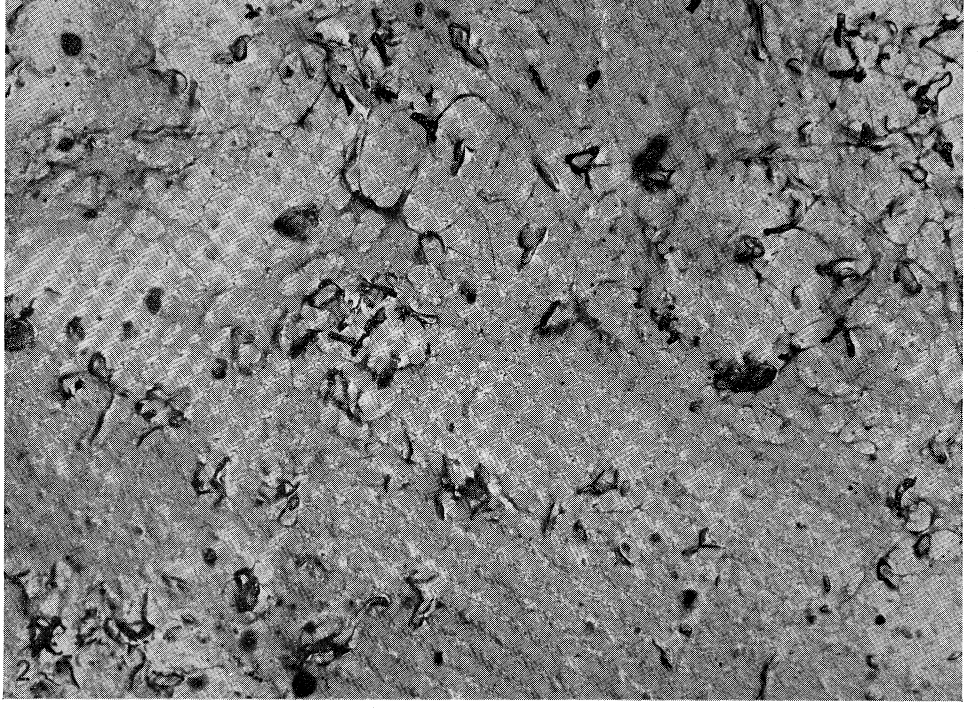

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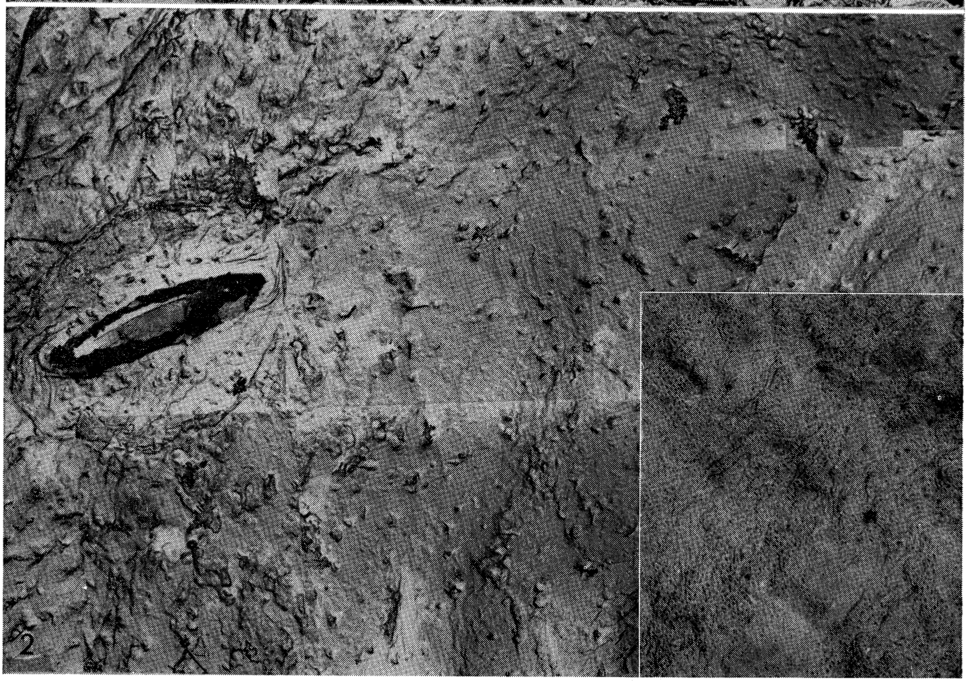

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