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HISTOLOGY OF PUSTULE FORMATION AFTER INOCULATING LEAVES OF PHASEOLUS WITH UROMYCES APPENDICULATUS

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SUMMARY

Histological studies of bean rust colonization revealed that mesophyll colonization accelerates abruptly on the sixth day following inoculation corresponding to (a) the time of development of the first visible symptoms and (b) the often reported manifold increase in respiration of diseased leaves. There is an accompanying hypertrophy of mesophyll cells. These observations are considered as further evidence that much of the respiratory increase may be ascribed to the fungus but they do not discount host contributions.

With scanning electron micrography and standard histological techniques, new observations made on the early stages of pustule development cast doubt on the (assumed) role of tactile pressure in uredial opening.

1. INTRODUCTION

In the literature there are many references to the changing metabolism of leaves inoculated with powdery mildews and rust fungi. Usually rates of photosynthesis are decreased while those of respiration are increased, the amounts of carbon dioxide respired increasing logarithmically and commonly reaching maxima six days after inoculation (ALLEN 1959, ALLEN & GODDARD 1938). In the many studies of respiration there has been a continuing problem when attempting to separate the contributions made by host and pathogen, the problem being greater with the relatively deeply penetrating rust fungi than with the mostly superficial powdery mildews. It is generally assumed when fungal attack increases rates of respiration that amounts of dry matter will decrease but, unexpectedly, ZAKI & MIROCHA (1965) found, after inoculating beans with *Uromyces appendiculatus* (*U. phaseoli*), that increased rates of respiration ($\times$ 2.5 on the fifth day after inoculation) were associated with dry weight increases. As the latter were not associated with concomitant increases in the host's rate of photosynthesis, they suggested that the greater dry weights might be attributable, at least partially, to the presence of rust mycelium and/or the accumulation of host material sequestered by the pathogen. They also noted (MIROCHA & ZAKI 1966) starch accumulation at about the time of sporulation. The starch soon disappeared but the increased weight persisted.

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To aid the interpretation of the physiological effects noted by Zaki and Mirocha and to further our understanding of infection processes, a detailed analysis was made of the sequence of changes of internal and external morphology occurring as pustules developed after inoculating *Phaseolus* plants with *U. appendiculatus*. These changes, some observed with the aid of a scanning electron microscope, are described in this paper.

2. MATERIALS AND METHODS

Seeds of *Phaseolus vulgaris* L. "Pinto III" were sown one per 7.5 cm plastic pot containing an unsterilized soil:sand:peat mixture (1:1:1) limed to a pH of 6.5–7.5. The pots were placed in water and the soil allowed to become saturated when they were drained and put in trays, covered with plastic sheeting and incubated in laboratory conditions for three days. Subsequently they were transferred to a growth chamber with 12 hr days (c. 1×10^4 ergs/cm^2/sec), the temperatures during light and dark periods being 21° and 16°C, respectively. Seven days later primary leaves of intact plants were sprayed with water suspensions of uredospores of *Uromyces appendiculatus* (Pers) Unger (*Uromyces phaseoli* (Pers.) Wint.) after which they were kept in a dark, moisture-saturated chamber at 15–20°C for 20–24 hrs before being returned to the growth chamber conditions already detailed.

For histological studies, small pieces (c. 1 mm^2) of leaf were fixed in either a 1:4:5 mixture of tertiary butanol:95% ethanol:water or Randolph's modified Navashin fluid ("Craf"), and then dehydrated in an ethanol:tertiary butanol series, embedded in paraffin and sectioned at 10 μ thickness. The material fixed in butanol:ethanol:water was stained with safranin and fast green; safranin, crystal violet, and orange G were used for material fixed in "Craf".

Scanning electron micrographs were taken of fresh material using a JSM-1 instrument with a 25 kv beam. To prolong the period of examination before tissues were distorted by the vacuum conditions of the microscope, differing coating procedures were tested. Not unexpectedly these proved to be of no advantage, since they also entail the use of vacuum. In the event photography of uncoated fresh material was begun as quickly as possible after insertion into the microscope and completed before surface reflectivity to the electron beam was lost.

3. RESULTS

Rust mycelium was not found within leaf tissues until four days (96 hrs) after inoculation (*fig 1a, b*) and was sparsely distributed but more readily detected after five days (120 hrs) (*fig 2*). Twelve hours later mycelium almost filled the large intercellular spaces of the spongy mesophyll (*fig 3*). Fungal stroma became conspicuous beneath the leaf epidermis by 144 hrs (*fig 4*). Later on the seventh day uredospores started to form.

As the spore mass (uredium) developed, the leaf epidermis separated from

underlying fungal and host tissues (*fig 5a*). This process of separation sometimes continued in pustules developing on the lower (abaxial) surfaces of leaves to form domes extending c. 15 μ beyond the limits of the surrounding epidermis (*fig. 8a, 9a*). On the upper surfaces domes invariably developed, usually reaching a height of 81 μ (mean of measurements made on ten replicate pustules), the epidermal cells being simultaneously distorted (*fig. 8a, b*).

These domes, which correspond to the fleck stage of macroscopic pustule development, and which are not considered to be artifacts of our methods of fixation, show clearly in the series of scanning electron micrographs (*fig. 5b, c*; *fig. 7a, b*). Cross sections indicate that the space between spores and the host's epidermis sometimes measures 28 μ (mean of 15 upper surface pustules). As the relatively few and small domes developed on the lower surfaces of leaves, the epidermal cells remained virtually unchanged but on the upper leaf surfaces the epidermal cells enlarged from 28.5 × 18.7 μ to 43.4 × 23.8 μ four and six days after inoculation, respectively (*figs. 4, 6, 9a*). On uninfected areas the comparable epidermal cells measured 27.9 × 13.6 μ on the sixth day.

Extreme distension and morphological aberration of epidermal cells was observed in scanning electron micrographs (*figs. 5, 7*). Cracks began to develop in the host's epidermis overlying developing infections after six and seven days (*fig. 9a, b*) and as these extended, pressure, from the developing mass of uredospores, seemed to push back the separated layer of epidermal cells with the production of a typical uredium with its spores protruding outwards and beyond the surrounding unaffected host tissues (*fig. 10a–d*).

In the period five to seven days after inoculation, mycelium of *U. appendiculatus* developed rapidly in the mesophyll with associated changes in the dimensions of palisade cells and of paradermal diameters. In uninfected areas of *Phaseolus* leaves, palisade and spongy mesophyll cells are both separated by intercellular spaces, these being particularly large among the former (*fig. 11a*). In colonized leaves, intercellular spaces are virtually absent unless occupied by mycelium (*fig. 11b*), there being a suggestion that infection increases the numbers of spongy mesophyll cells. This observation made on leaf sections was not corroborated when staining techniques for detecting mitotic divisions were applied. At this stage therefore the mesophyll distortions causing the leaf thickening illustrated in *fig. 12* are attributable to hypertrophy but not to hyperplasia.

4. DISCUSSION

Amounts of carbon dioxide respired by rust and mildew-infected leaves increase greatly four to six days after inoculation (ALLEN 1959, ALLEN & GODDARD 1938, ZAKI & MIROCHA 1964). Controversy has developed as to whether observed increased metabolic activity is due largely to increased host or parasite activity. It has been demonstrated that the respiration increases are much larger at the centre of vigorously sporulating rust pustules than in adjacent host tissue (SAMBORSKI & SHAW 1956). Radioisotope techniques demonstrated

accumulation of labelled material in the infection area (DURBIN 1965) and work of greater resolution located almost all of it in the sporulating rust fungus (STAPLES & LEDBETTER 1958).

The rapid rise in respiration is associated with the "fleck" stage of rust pustule development but nowhere can there be found evidence associating the sharp respiratory increase at this time with simultaneously increased fungal development.

Reports by ZAKI & MIROCHA (1964, 1965) describe normal respiration of diseased (bean rust) plants on the fourth day after inoculation followed by a rapid two-and-a-half fold increase on the fifth day when flecks appeared. In feeding $^{14}CO_2$ to infected plants they reported localization in discrete circular areas on the fourth day before symptoms were visible and attributed this to dark fixation by the fungus. Dry weight increases in infected tissue coincided with the time of increase of respiration after the fourth day, and they suggest that part of the dry weight increase may be due to the presence of mycelium.

Our observations of morphology indicate that these changes in respiration and dry weight are associated in "Pinto" bean leaves with a similarly sudden increase in the amounts of *U. appendiculatus* mycelium.

The parallel nature of these two events should not, however, be taken to imply a simple cause and effect relation, hypertrophy of mesophyll cells as documented here being but one of possibly several indicators of a change in the host's metabolism.

YARWOOD & COHEN (1951) have discussed hypertrophy associated with bean rust infections including data on cell size obtained from chloral hydrate cleared leaves seven days after inoculation. The validity of their data is however in doubt because they describe and give cell measurements for a "second palisade layer" which *Phaseolus vulgaris* does not usually possess. Its formation was not triggered by *U. appendiculatus* in the present investigation.

Use of the scanning electron microscope has extended the detail obtained from traditional light microscopy. Its depth of focus and high resolution have enabled us to question the generally held belief that developing uredospores rupture the epidermis by tactile pressure alone. In the event, the overlying epidermis cracks before the developing spores are adpressed to it except near giant cells at the perimeter of developing uredia. The host's epidermis seems to act as a membrane which becomes inflated with the accumulation of possibly volatile metabolites which ultimately cause it to break ahead of contact with the spores themselves. The increasingly wide separation of epidermis from underlying tissues probably explains the changing coloration from green to light green to white as pustules (flecks) mature.

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A sequence illustrating the colonization of *Phaseolus* bean leaves by *Uromyces appendiculatus*.

Fig. 1. *a,b*. Mesophyll of *Phaseolus* bean leaves showing typically sparse hyphal invasion four days after inoculation with *Uromyces appendiculatus*.

Fig. 2. Five days after inoculation showing little change from fourth day.

Fig. 3. Five and one-half days after inoculation showing proliferation of hyphae.

Fig. 4. Six days after inoculation showing mesophyll and adaxial epidermal hypertrophy and early stromatal development.

Fig. 5. Six days after inoculation.
5*a*. Palisade hypertrophy, first sporophores, epidermal hypertrophy and separation.
5*b,c*. Scanning electron micrographs (SEM) of adaxial epidermis showing pre-uredial domes and distension of epidermal cells of dome.

Fig. 6. Hypertrophied ("giant") epidermal cell at edge of dome.

Fig. 7. Six/seven days after inoculation.
7*a,b*. Pre-uredial domes and giant cells at edges (SEM).

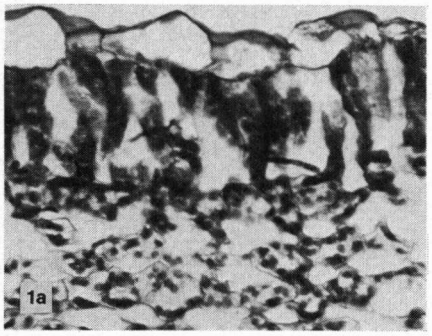
Fig. 8. Six/seven days after inoculation.
8*a*. Low power view showing differences between upper and lower epidermis.
8*b*. High-power view of upper epidermis showing separation of distended epidermal cells of dome from mature spores.
8*c*. High-power view of lower epidermis (inverted) showing same separation although dome is less pronounced.

Fig. 9. Seven days after inoculation.
9*a*. Low power view showing first crack in upper epidermis.
9*b*. SEM view of early stage of uredial opening.

Fig. 10. Seven/nine days after inoculation.
10*a*. Epidermal flange at edge of mature uredium.
10*b*. SEM micrograph of similar epidermal flange showing individual epidermal cells and free, mature spores.
10*c*. SEM micrograph of entire uredium.
10*d*. Low power view, comparable to 10*c*, showing exposure of spores above mean epidermal level.

Fig. 11. Development of hypertrophy.
11*a*. Normal mature mesophyll.
11*b*. Palisade and spongy mesophyll hypertrophy in pustule area.
11*c*. Spongy mesophyll hypertrophy and hyphal perfusion.

Fig. 12. Low power view of edge of infection locus allowing comparison of normal and hypertrophied tissues.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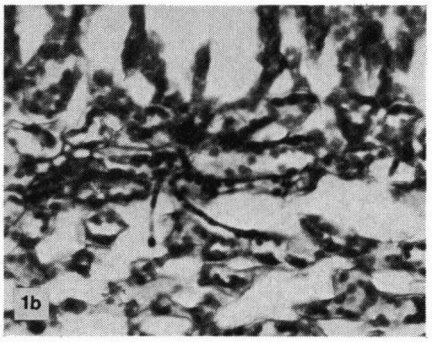
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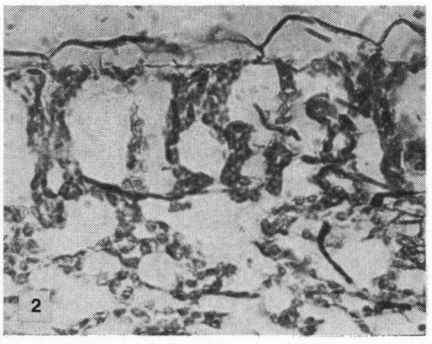
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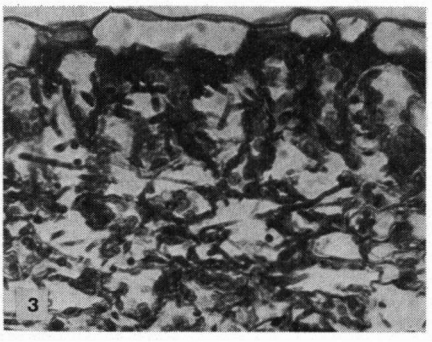
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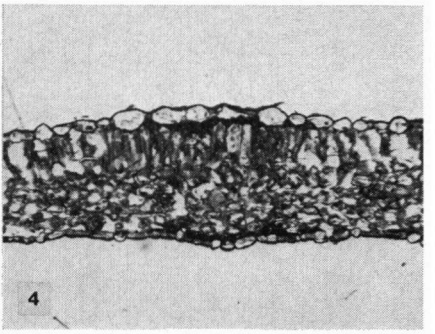
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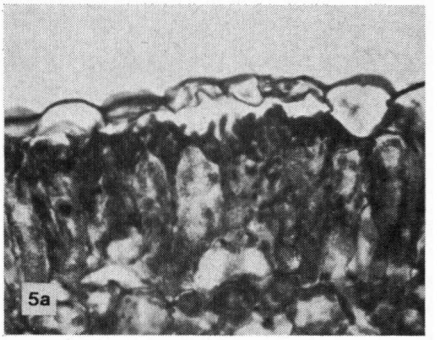
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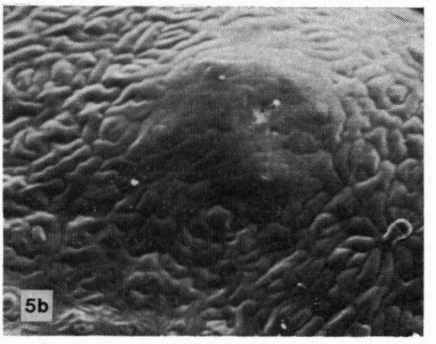
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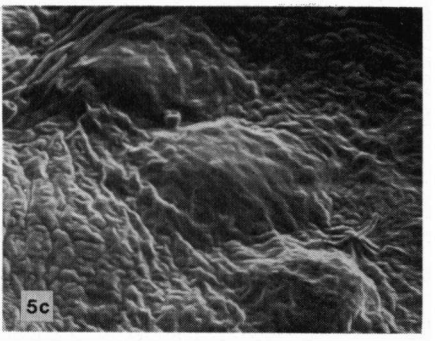
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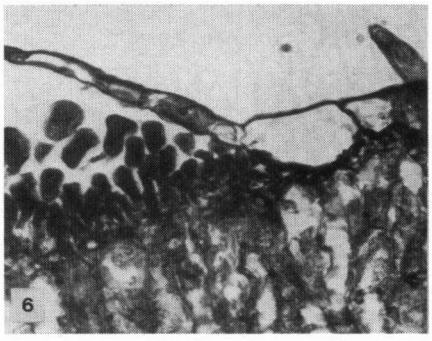
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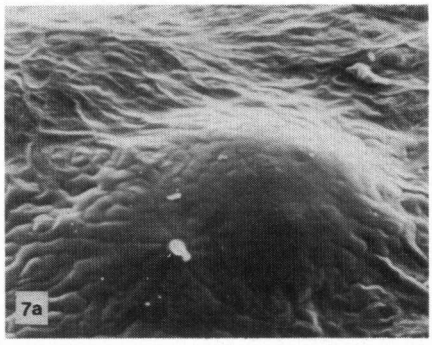
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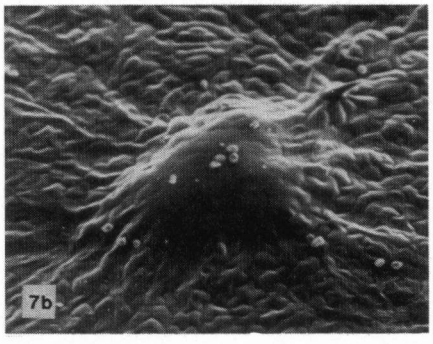
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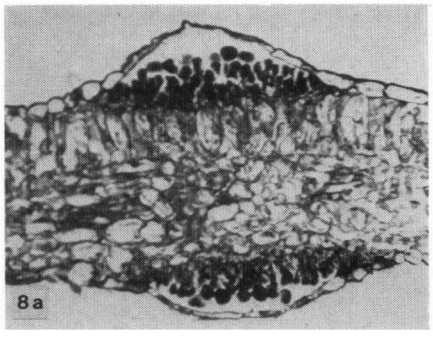
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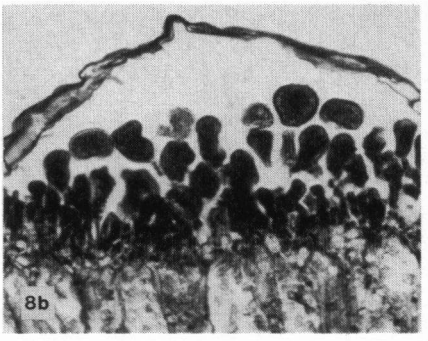
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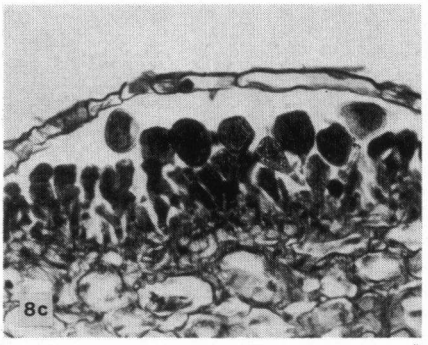
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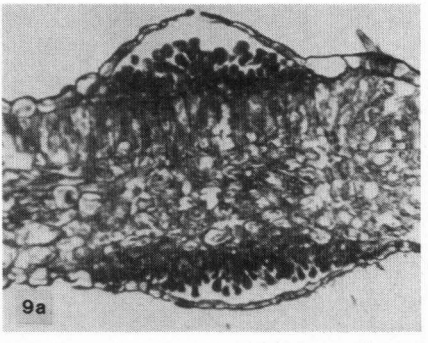
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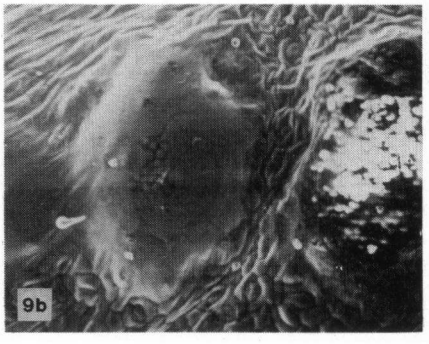
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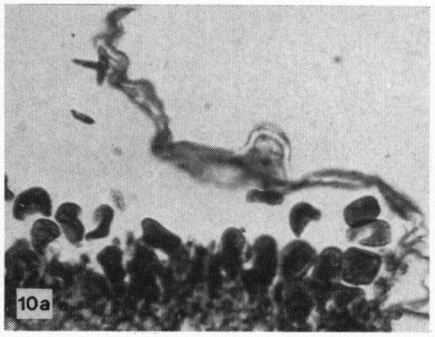
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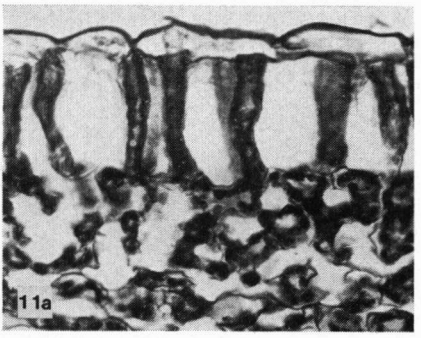
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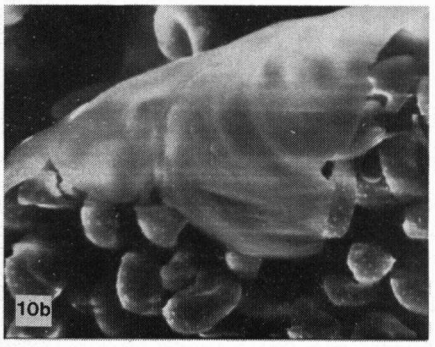
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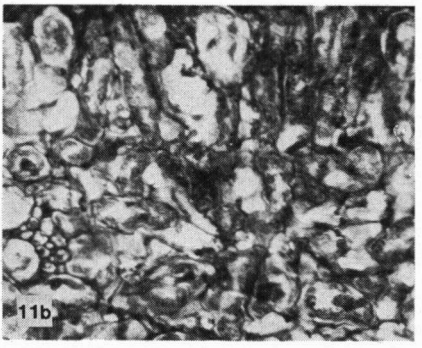
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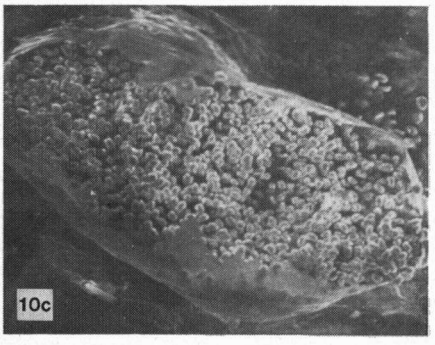
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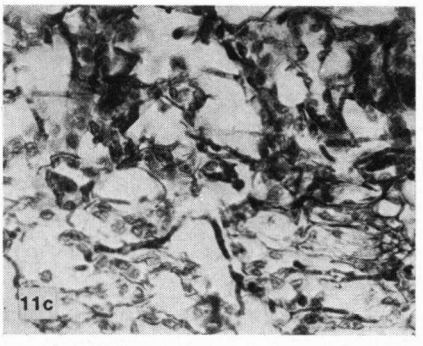
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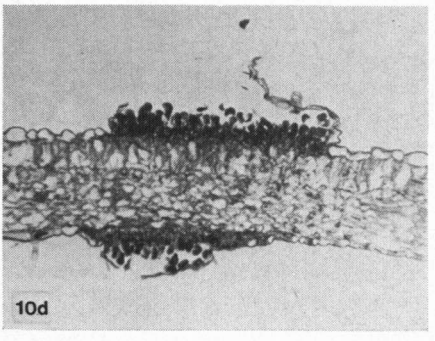
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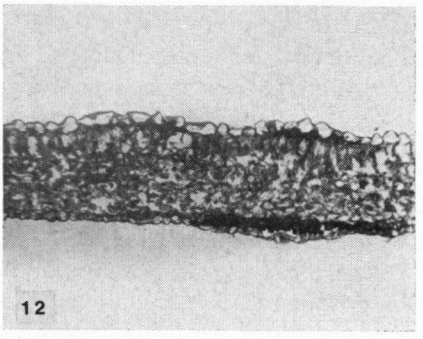
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