A MORPHOLOGICAL AND HISTOCHEMICAL STUDY OF THE BACTERIAL DEGRADATION OF WOOL FIBRES *IN VIVO*

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[*Manuscript received February* 21, 1961]

Summary

It has been previously shown that a microorganism, isolated from the contents of dermal cysts artificially produced in a Merino sheep, was capable of degrading wool fibres *in vitro*. This paper considers the degradation of wool fibres *in vivo* and describes structural changes and distribution of sulphydryl groups demonstrated by histochemical technique. The fibres are arranged in order of degree of degradation which is characterized by two mechanisms; reduction of –S–S– cross-linkages and proteolysis. The degradation proceeds to disintegration of macrofibrils.

I. Introduction

In a previous communication (Molyneux 1959) the characteristics of a microorganism capable of degrading wool fibres and the method of its isolation from the contents of an experimentally produced epithelium-lined cyst were described. The degradation of the wool fibres *in vitro* proceeded stepwise; the removal of intercellular material released free cortical cells and the removal of intracellular material released intracellular fibrils. Because of the production of pink pigment in the degradation of Merino wool fibres *in vitro* and because the microorganism was isolated from an experimental cyst, a comparison was made between the degradation of wool occurring in the cysts and the degradation of wool in "pink rot", a variety of fleece rot described by Waters (1932).

The removal, 56 and 83 weeks after implantation in a Merino sheep, of two experimental cysts in which the degradation of wool fibres had occurred *in vivo*, has made possible the examination of these fibres by electron-microscopy and staining techniques.

In this paper the fibres have been arranged in an order of degradation which is influenced by their bilateral structure. The bilateral structure of wool fibres is now well established. Horio and Kondo (1953) showed that the characteristic crimp of Merino wool fibres is related to the bilateral structure of the individual fibres. They showed that when wool fibres are dyed with a basic dye (janus green) the cortex is divided into dye-accessible (DA) and non-DA segments, the latter being situated on the inside of the curvature of the coiled or crimped fibres. They also showed that the DA segment is sensitive to weak alkali as evidenced by swelling and loss of birefringence. Mercer (1953) showed that, when fine Merino wool is supercontracted by heat and then treated with trypsin, there is preferential digestion of a segment of the

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fibre. This segment was named the orthocortex and corresponds to the DA segment of Horio and Kondo. The segment resistant to enzymic digestion, the paracortex, corresponds to the non-DA segment. Also Fraser and Rogers (1953) described the division of wool fibres into S (soft) and H (hard) segments, corresponding to the orthocortex and paracortex respectively, as judged by the marked susceptibility of the S-segment to reduction with alkaline thioglycollate.

II. Materials and Methods

Before surgical removal of the experimental cysts, a punch biopsy of the cyst walls was made. At this time a sample of intracystic wool fibres was removed and subsequently fixed in 1% trichloroacetic acid in 80% alcohol. The whole cysts were then removed and fixed in buffered 10% neutral formalin. Paraffin-embedded sections, 8–10 μ in thickness, were prepared and representative sections were stained with haematoxylin and eosin for routine examination.

The bilateral segmentation of the fibres was examined by dyeing the sections with 0·1% methylene blue in 0·03M phosphate buffer at pH 7·4 for 30 min at 100°C, as described by Fraser and Rogers (1955*a*).

The location of protein-bound sulphydryl groups was demonstrated by the use of dihydroxydinaphthyldisulphide (DDD), a naphthol derivative, as described by Barrnett and Seligman (1952) and Barrnett (1953). As controls for the DDD reaction some sections were stained with tetrazotized diorthoanisidine alone and in others blockade of sulphydryl groups was obtained by treatment with 0·1M *N*-ethyl maleimide in phosphate buffer at pH 7·4 for 6 hr at 37°C. Sections of normal Merino skin fixed in formol saline and samples of normal Merino wool, unfixed and fixed in trichloroacetic acid–alcohol, were used as additional controls in the staining methods.

Samples of intracystic fibres, previously fixed in neutral buffered formalin, were prepared for electron-microscopy. Fibres, taken from area *a* as shown in Plate 1, Figure 1, were treated with 1% osmium tetroxide in acetate–veronal buffer at pH 7·4 for 3 hr and then embedded in "Araldite" using a modification (Rogers, personal communication) of the method described by Glauert, Rogers, and Glauert (1956). To obtain additional contrast the mounted "Araldite" sections were treated for 30 sec–2 min with lead hydroxide before microscopy, as described by Watson (1958) and Peachy (1959). A sample of wool undergoing bacterial degradation *in vitro* (Molyneux 1959) was also prepared for electron-microscopy. Partially degraded wool fibres consisting of free cortical and cuticle cells at various stages of degradation were collected from a stock Merino wool–salts culture by centrifugation, stained in osmium tetroxide as above, and embedded in "Araldite".

III. Results

As shown in Plate 1, Figure 1, the cysts were packed with wool fibres which when examined were at various stages of degradation. Because wool fibres continued to grow into the cyst cavity (Molyneux and Lyne 1961), the greatest breakdown of fibres, as would be expected, occurred towards the centre of the cyst, where the oldest

fibres lie. The peripheral fibres, those adjacent to the cyst epithelial lining, showed the least degradation and, on examination with histological stains, appeared to be normal. This temporal arrangement of fibres within the cyst cavity, together with alteration in structure and histochemical staining, has allowed the intracystic fibres to be placed in an "order of degree of degradation".

(*a*) *Staining Reactions*

(i) *Haematoxylin and Eosin.*—Evidence of degradation in the fibres was seen in preferential staining of some fibres with eosin. The differential uptake of dye indicated the existence in these fibres of two segments, the greater segment alone being stained. In the eosinophilic segment the cellular outlines of cortical cells were distinguished, while the smaller segment remaining unstained showed no evidence of its cellular structure (Plate 1, Fig. 2). Some fibres stained completely with eosin, while others were completely unstained. The debris of fibre degradation, free cortical cells, and fibrils stained variously.

(ii) *Methylene Blue.*—In order to correlate the abovementioned segmental staining with the known bilateral structure of wool fibres, sections were dyed with methylene blue. Fibres which remained unstained with eosin, and showed no structural change, exhibited preferential uptake of basic dye and were observed to consist of two segments, the larger of which stained lightly except for cortical cell outlines and nuclear remnants which stained more heavily, as described by Fraser and Rogers (1955*a*).

Fibres in which partial degradation had occurred also showed segmental staining with methylene blue; the degraded larger segment which in normal fibre stained with eosin now stained heavily with methylene blue; the smaller intact segment stained lightly (Plate 2, Fig. 1). Fibres in which the degradation extended through their whole thickness stained with methylene blue, although even in many of these the segmental staining was apparent in that the larger segment stained more heavily. The uptake of dye was less as the degradation of the fibres increased. From these observations the initially degraded segment of the fibres was considered to represent the orthocortex.

(iii) *Location of Sulphydryl Groups.*—Examination for the presence of sulphydryl groups showed that, whereas the majority of intracystic fibres stained heavily, only nuclear remnants and small scattered particles stained in normal control fibres. It was found that intracystic peripheral fibres stained blue (high concentration of sulphydryl) while the more central fibres stained predominantly red (lower concentration of sulphydryl). This has been interpreted as a depletion of sulphydryl-containing material from the fibres as degradation proceeds.

A temporal arrangement of fibre degradation may be conveniently illustrated by the following stages:

Stage A: The fibre consisted of two unequal segments. In the larger segment the cell outlines and nuclear remnants stained. The cuticle was intact.

Stage B: (cf. Plate 2, Fig. 2). In the orthocortex the periphery of the cortical cells and nuclear remnants stained more heavily blue. A number of cortical cells stained completely blue while others were only partly stained; the stain appeared to penetrate the cells from the intercellular region. At this stage isolated cells, which were unstained except for the nuclear remnant, were seen in the orthocortex. In the paracortex the nuclear remnants stained more heavily than in (A). The cuticule was intact.

Stage C: (cf. Plate 3, Fig. 1). The larger segment stained heavily blue, so that cortical cell outlines were obscured. Isolated cells may remain unstained. Irregular red-stained areas (*R*) occurred. In the smaller segment cortical cell outlines were visible and stained a faint blue. The cuticle was intact.

Stage D: (cf. Plate 3, Fig. 2). The cortical cells of the greater segment stained uniformly red and were often separated from one another; the intercellular material and nuclear remnants were not visible. The appearance of the smaller segment varied. For example: (*a*) there may have been no structural change and only the nuclear remnant stained blue; (*b*) intercellular material, nuclear remnants, and cortical cells may have stained blue; and (*c*) intercellular material and nuclear remnants may not be visible and the cortical cells were unstained. The cuticle, when present, generally had ruptured on the side covering the orthocortex (fibre D, Plate 3, Fig. 2) or it was absent (fibre D_1, Plate 3, Fig. 2).

Stage E: (cf. Plate 3, Fig. 1). The cortical cells of the whole fibre stained red, the intercellular material and nuclear remnants being absent. The cuticle may be intact, may have ruptured and partly lifted from the fibre, or be entirely absent.

Stage F: (cf. Plate 3, Fig. 2). In the intracystic debris, free cortical cells, fibrils, cuticular cells, and keratin flakes from the stratum corneum could be recognized. Free cortical cells stained red or were unstained.

(*b*) *Electron-microscopy*

An electron-micrograph of a degraded fibre such as D_1 in Plate 3, Figure 2, is shown in Plate 4, Figure 1. There is an absence of cuticle and of intercellular, intermacrofibrillar, and nuclear material. The macrofibrils remain arranged so that cortical cell outlines can be distinguished.

Plate 4, Figure 2 shows an electron-micrograph of an isolated cell (stage F of degradation). Some macrofibrils are fragmented and there is an absence of intermacrofibrillar material. Higher magnification in Plate 5 reveals a microfibrillar pattern. A conspicuous feature is the fragmentation of the macrofibrils which have an ill-defined periphery. Just beneath this periphery there appears to be a band of increased electron density. Within the macrofibrils are circumscribed areas of in-

creased density which are demarcated from the surrounding macrofibrillar structure by an area of decreased density in which the fibrillar pattern is interrupted. In Plate 5, two such areas, *a* and *b*, are prominent. A portion of area *b* has apparently been lost during preparation. It is probable that these areas of increased electron density represent areas of initial degradation. Fibres degraded *in vitro* are shown in Plate 6, Figure 1. Central degradation of the macrofibrils is prominent. Plate 6, Figure 2, shows the appearance of the cuticle which had separated from an intracystic fibre. At the outer border of the cuticle a dense band can be seen in the exocuticle. Degradation has occurred involving partial loss of structure in the region of the exocuticle and endocuticle of the cell.

IV. Discussion

By the application of histochemical techniques to intracystic wool fibres, a series of stages in fibre degradation has been revealed. On the basis of distribution and relative concentration of sulphydryl groups, together with a consideration of structural changes, the probable sequence of these stages of degradation has been determined.

Control (normal) fibres when stained by the DDD procedure did not react, except in so far as nuclear remnants and various rodlets stained blue. In contrast, as degradation of intracystic wool fibres proceeds the initial staining for sulphydryl groups occurs in the intercellular region of the orthocortex (stages A and B), next the entire orthocortex begins to stain heavily for –SH groups, indicating the reduction of –S–S– linkages (stage C) while the entire fibre is stained in stage E. The decrease in sulphydryl staining intensity, from blue to red, as degradation proceeds indicates a progressive loss of sulphydryl groups from the fibre. The staining of the intercellular region between the cortical cells occurs before structural change in the fibres is apparent by light-microscopy. It is possible, however, that this staining may give a false location of sulphydryl groups as reaction products from the cell contents could diffuse into intercellular spaces as a result of digestion of the intercellular membrane complex. At stages D and E marked structural changes are shown such as rupture and loss of cuticle and loss of intercellular material and nuclear remnants, which result in the release of cortical cells. Electron-micrographs show further stages of degradation in which macrofibrils are freed by the removal of intermacrofibrillar material. The macrofibrils then begin to disintegrate and gradually digest.

Geiger *et al.* (1941) demonstrated that reduction of –S–S– linkages in wool increased its susceptibility to digestion by proteolytic enzymes. Fraser and Rogers (1953) demonstrated the susceptibility of the orthocortex of Merino wool fibre to reduction with sodium thioglycollate, while Mercer, Golden, and Jeffries (1954) showed that the orthocortex is preferentially digested by pepsin following reduction with thioglycollic acid. This pattern of degradation is seen in the initial reduction of –S–S– linkages in the orthocortex of intracystic fibres. Moreover, the contrast obtained in electron-micrographs of degraded macrofibrils stained with osmium tetroxide followed by lead hydroxide (Plate 5) is similar to that shown by Merino

fibres previously reduced with 0·5M thioglycollic acid and then stained with osmium tetroxide (Rogers 1959*b*) and suggests that some reduction of the cystine of the sulphur-rich matrix between the microfibrils has occurred.

The partially degraded intracystic fibres (Plate 4, Fig. 1; Plate 6, Fig. 2) are similar to free cuticle and cortical "cells" released from wool fibres following prolonged tryptic digestion (Mercer, Farrant, and Rees 1955). The fibre components digested include the endocuticle, intercellular material, nuclear remnants, and intermacrofibrillar material. The digestion of the latter component releases individual macrofibrils. Following oxidation and extraction of wool fibres with dilute ammonia, the abovementioned fibre components remain, and have been termed "non-keratinous" (Mercer 1953, 1955; Rogers 1959*b*). Structural degradation of fibres initially occurs in the orthocortex which, in comparison with the paracortex, has a greater amount of intermacrofibrillar material (Rogers 1959*a*, 1959*b*) and is more susceptible to proteolytic attack.

Circumscribed areas of increased electron density were observed within macrofibrils from degraded intracystic fibres (*a* and *b* in Plate 5). Similar areas have been observed by Rogers (personal communication) in normal wool fibres, reduced wool fibres treated with enzymes, and particularly in porcupine quills. These areas, which could be considered as areas of faulty organization, may be initially more susceptible to digestion than the more organized parts of the macrofibril. The presence of such areas may be responsible for the central degradation of macrofibrils shown in Plate 6, Figure 1. In Plate 6, Figure 2, the dense band seen in the exocuticle corresponds to the region of the exocuticle in human hair which is particularly resistant to tryptic and keratinolytic attack (Birbeck and Mercer 1957).

Thus evidence is presented that the degradation of intracystic fibres is characterized by two mechanisms: reduction of –S–S– linkages and proteolysis. The sequence of fibre degradation, determined by structural changes and the distribution of sulphydryl groups, is described while electron-microscopy reveals the final disintegration of the macrofibrils.

V. Acknowledgments

The author is indebted to Dr. G. E. Rogers, Division of Protein Chemistry, C.S.I.R.O., for his helpful advice and criticism during this work, and to Associate Professor D. A. Cameron, University of Sydney, for the preparation of the electronmicrographs.

Through the courtesy of Professor B. T. Mayes, Director, Queen Elizabeth II Research Institute for Mothers and Infants, University of Sydney, facilities were made available for electron-microscopy.

Professor K. W. Cleland, University of Sydney and Dr. G. E. Rogers kindly read the manuscript.

The author is also indebted to the National Health and Medical Research Council for a grant to enable this work to be carried out.

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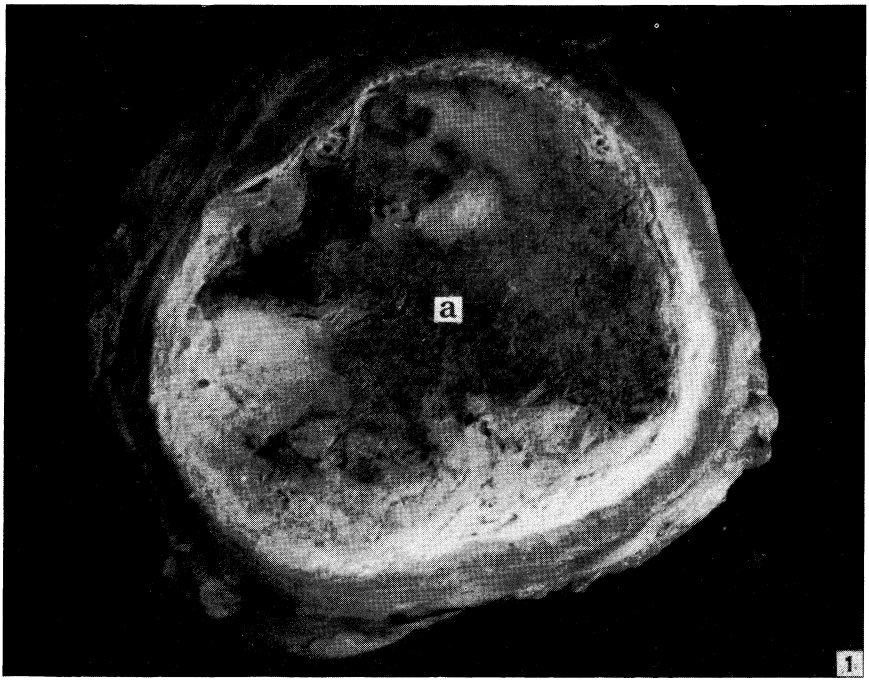
Explanation of Plates 1–6

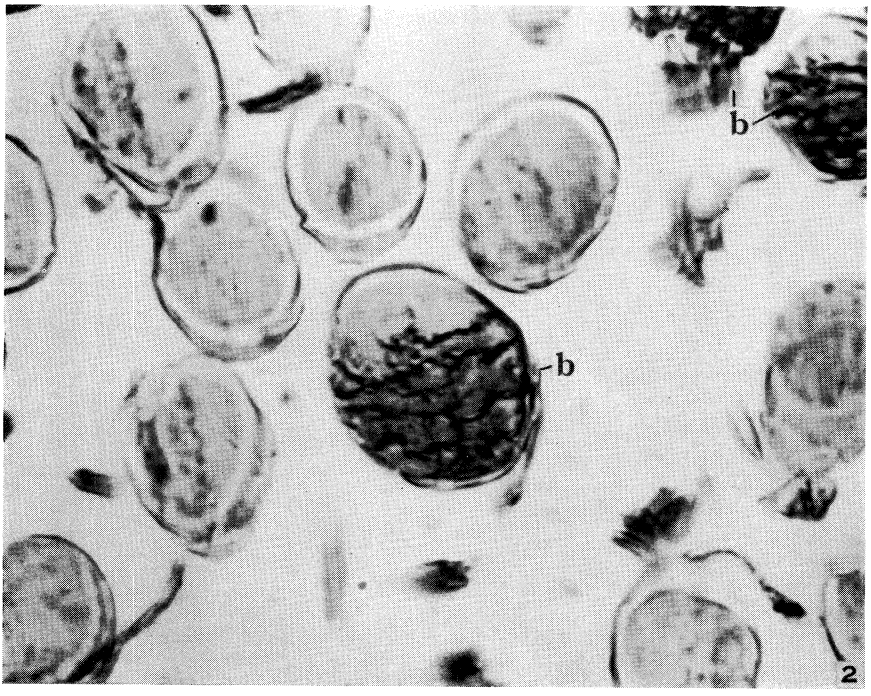
Plate 1

Fig. 1.—Cross section of an experimental dermal cyst removed 83 weeks after implantation. Degraded wool fibres appear dark while relatively normal fibres are situated at the periphery adjacent to the cyst wall. ×3·7. *a*, Area from which fibres were taken for electron-microscopy studies.

Fig. 2.—Intracystic fibres stained with haematoxylin and eosin. *b*, Fibres showing structural change and which are stained with eosin. ×950 approx.

DEGRADATION OF WOOL IN VIVO





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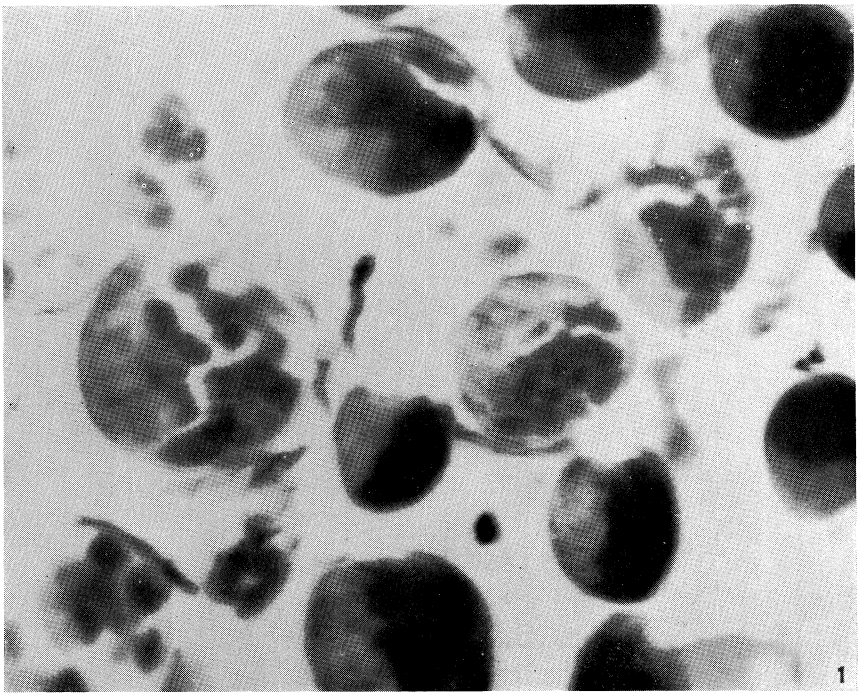

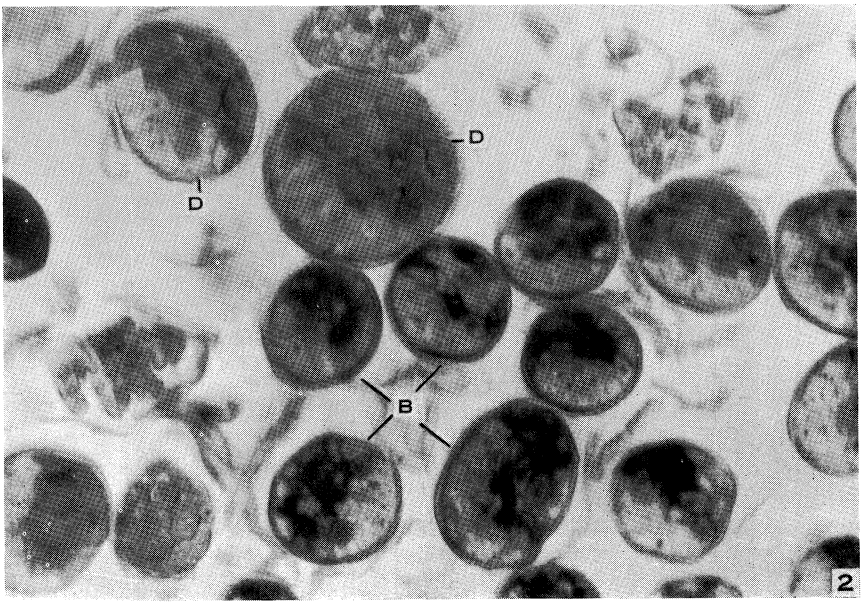

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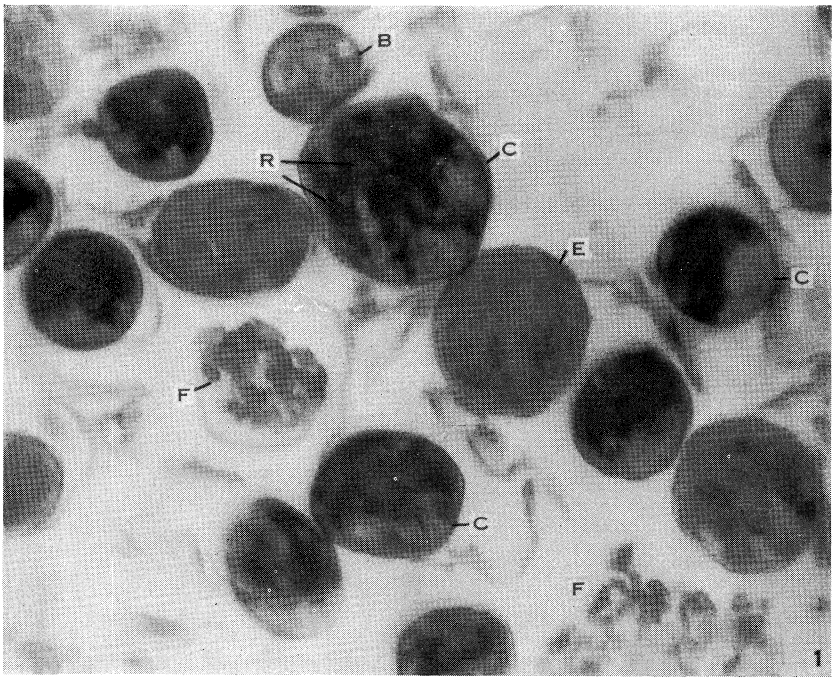

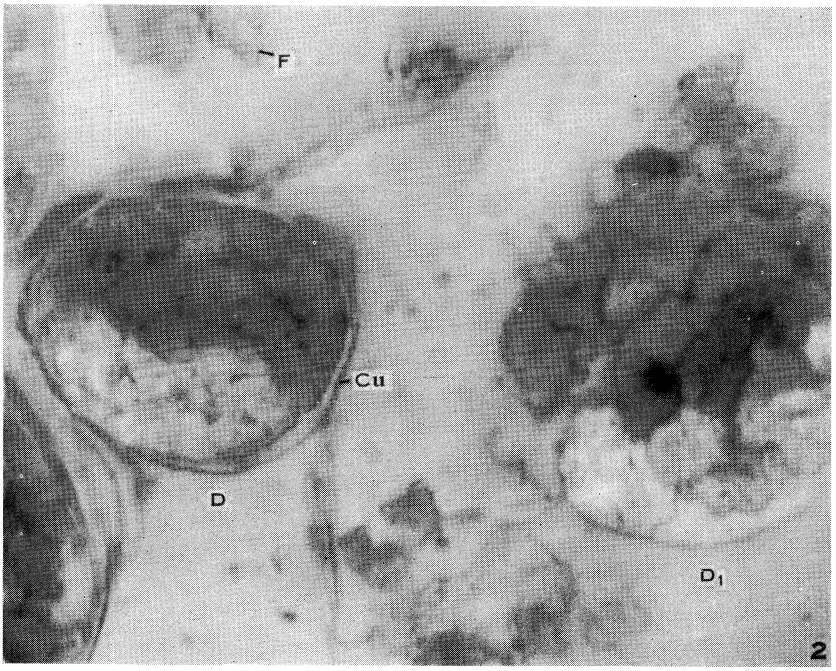

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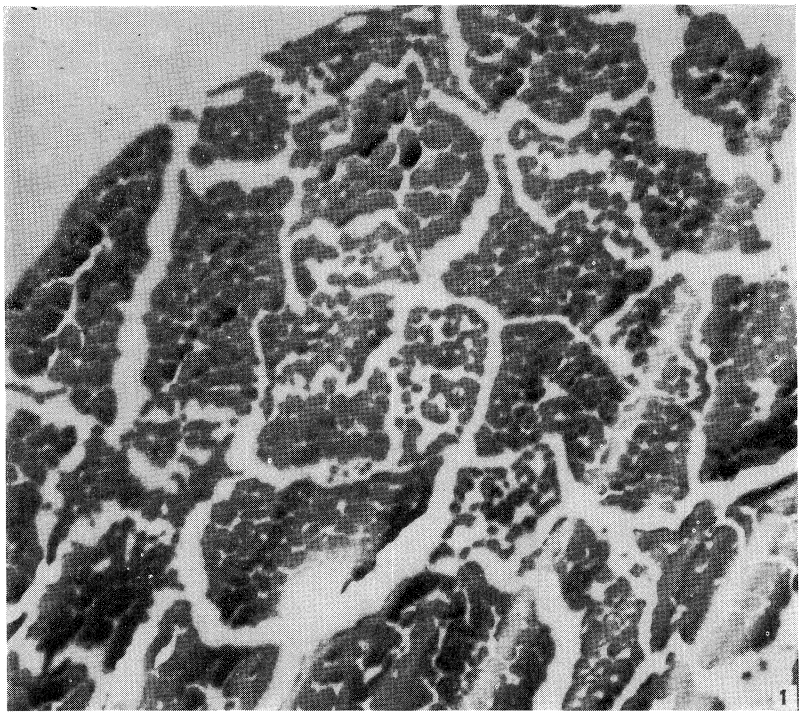

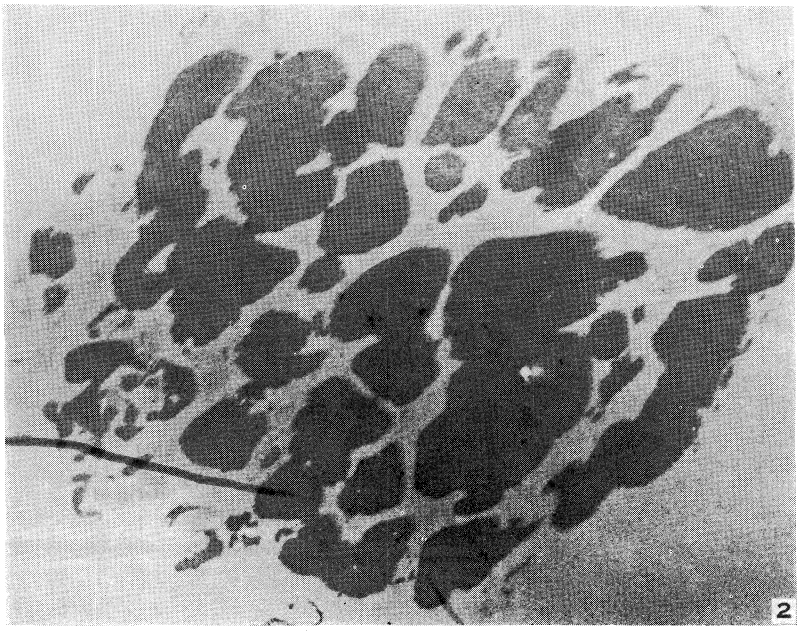

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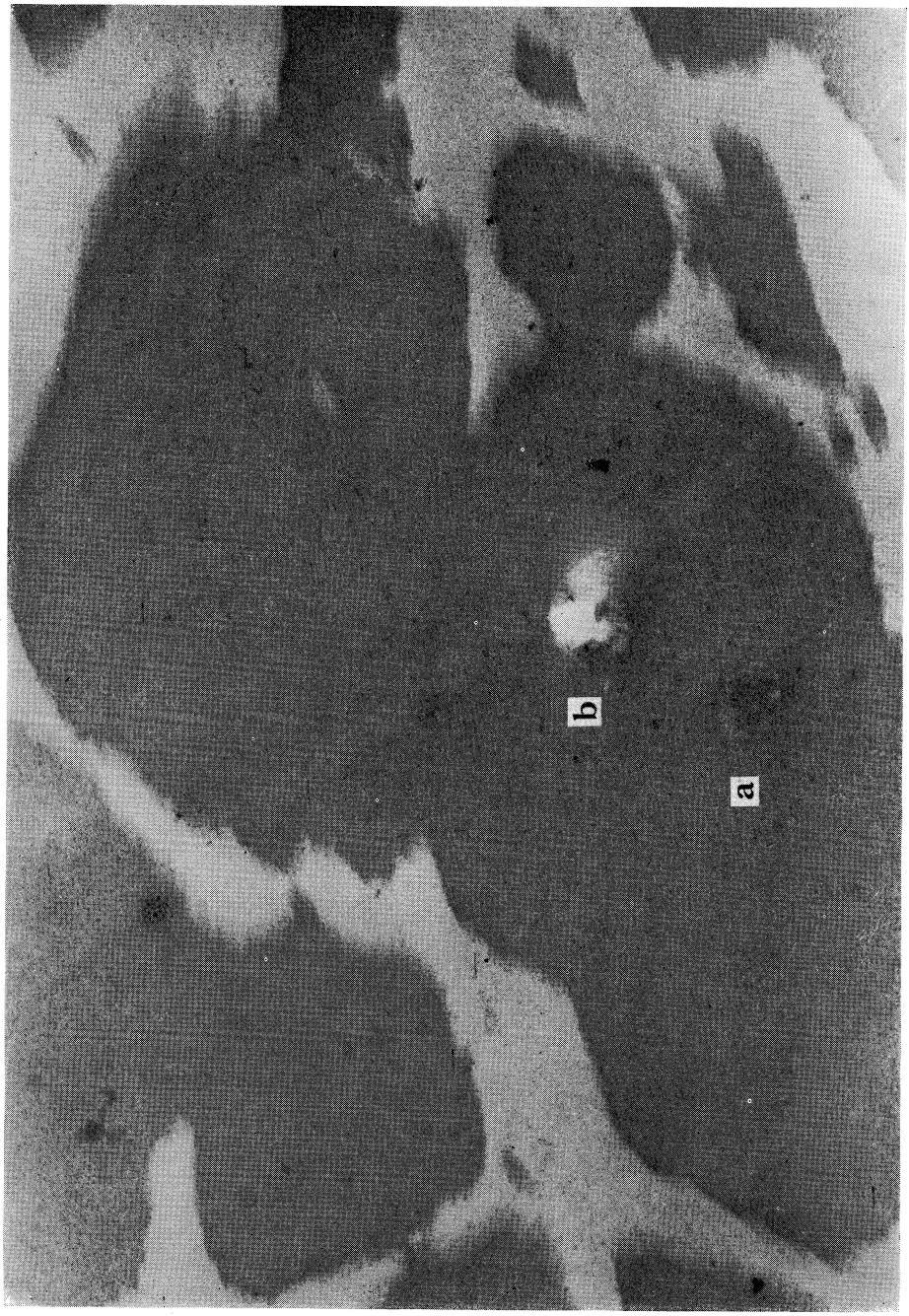

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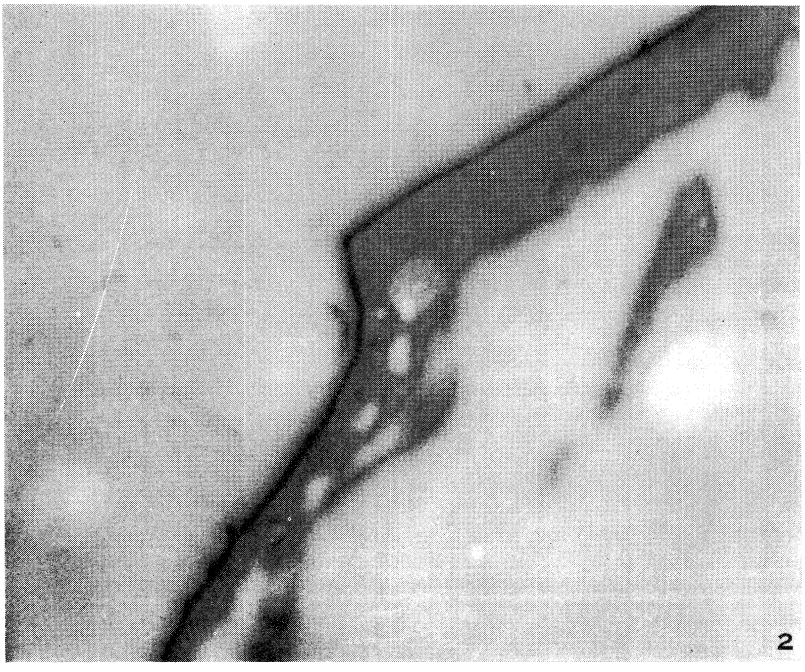

PLATE 2

Fig. 1.—Fibres stained with methylene blue showing heavy staining of the orthocortex. Initial degradation of the orthocortex is shown by the release of cortical cells. ×760 approx.

Fig. 2.—Fibres stained for sulphydryl groups showing stages B and D of degradation. ×760 approx.

PLATE 3

Fig. 1.—Fibres stained for sulphydryl groups showing stages B, C, E, and F of degradation. ×760 approx. *R*, irregular red-stained areas.

Fig. 2.—Fibres stained for sulphydryl groups showing stages D and F of fibre degradation. The cuticle has lifted from the fibre in D and is absent in D_1. The paracortex of fibre D_1 is unstained and intercellular material is absent. F shows free cortical cells. ×1800 approx.

PLATE 4

Fig. 1.—Electron-micrograph of fibre in stage D_1 of degradation. Cuticle, nuclear remnants, and intercellular and intermacrofibrillar material are absent. Cortical cell form can still be distinguished. ×6000.

Fig. 2.—Group of macrofibrils showing evidence of disintegration. ×21,000.

PLATE 5

Higher-power magnification of macrofibril from Plate 4, Figure 2, showing prominent microfibrillar pattern. Partial fragmentation has occurred. Areas *a* and *b* suggest areas particularly susceptible to degradation within the macrofibril. ×84,000.

PLATE 6

Fig. 1.—Electron-micrograph of macrofibrils degraded *in vitro*. Fragmentation is advanced with prominent central degradation. ×56,000.

Fig. 2.—Electron-micrograph of cuticle from intracystic fibre showing a dense band in the outer region of exocuticle and partial degradation of the exo- and endocuticle. ×32,000.