SELECTIVE DESTRUCTION OF THE EXINE OF POLLEN GRAINS*

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ABSTRACT

Treatment of *Nuphar, Betula, Epilobium, Zauschneria*, and *Malva* pollen with (1) KOH, (2) chlorine producing solutions, (3) the acetolysis mixture of Erdtman, or (4) hot water, resulted in migration or extraction of substances that stain positively for protein and acidic polysaccharide and are darkened by osmium tetroxide, suggestive of lipoidal substances. These extractable substances are apparently located within the interstices of the exine matrix. When treatments 1-3 were applied successively, in any order, or pollen was exposed to 20% chromic acid, exine matrix was removed selectively from exines. The exine remnants then appeared variously filamentous, fibrillar, or lamellar. As exine matrix is reduced in volume and filamentous structure is revealed stainability and production of a reddish colouration in sulfuric acid becomes extraordinary. On further destruction of exine matrix all stainability, including reactivity to sulfuric acid, is extinguished. The exine remnants are still filamentous in appearance and they embody most of the features of specific exine form. Since these unstainable remnants continue to be autofluorescent under ultraviolet excitation, they are assumed to consist of sporopollenin. We consider that as exine matrix (sporopollenin) is degraded stain reactive macromolecules encapsulated within sporopollenin are progressively exposed, accounting for the extreme stainability of our treated exines and some fossil exines. Although staining for protein and darkening by osmium is elevated, emphasis is given to staining for acidic groups at low pH since acidic polyanions are not considered as part of hypothetical structures for the sporopollenin matrix. After stainability is extinguished, further treatment, even with water, may cause exine remnants to swell and then disintegrate, after which autofluorescent filaments are observable in the sediment following centrifugation suggesting that sporopollenin remains after exines have disintegrated. Swelling and disintegration could be prevented by methods commensurate with stabilization of polysaccharides, suggesting application of our data in methods for extraction of exines from sediments. Our interpretation is that after the volume of sporopollenin in exines is reduced below a critical level, reactive macromolecules within the exine are no longer protected by envelopment in sporopollenin and are subject to destruction or extraction. We consider that the most important aspect of our study concerns the probability that the macromolecules embedded within sporopollenin of exines of pollen grains and spores will have, as a result of their apparent origin as plasma membrane glycocalyx components, a specific chemical composition. The utility of unique macromolecules protected by encapsulation in sporopollenin so as to withstand unbelievably severe conditions, e.g. fossilization, has implications of unheard proportions.

INTRODUCTION

Our experiments were stimulated by discussions with ROBERT H. TSCHUDY and GUNNAR ERDTMAN who had observed a pronounced swelling of exines of some pollen grains and spores following successive treatment by acetolysis—chlorination—KOH. We considered that the swollen exine might be employed for analysis of exine fine structure analogous to the studies of BAILEY AND KERR (1935) with swollen primary and secondary cell walls.

In preliminary experiments we found that relatively brief treatment by the battery of reactions produced a dramatic increase in basophilia and osmophilia, often accompanied

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†In memorial to Dr. Botjah Prijanto who was reported to have been killed in 1967 as the result of an automobile accident near his home in Indonesia. Botjah was a devoted scientist and a noble man.

by extreme stickiness of exines, whereas exine stainability tended to be extinguished by the longer periods of treatment which produced exine swelling. We decided to study the former phenomenon because, not only were swollen exines difficult to visualize except with phase contrast and under ultraviolet, but the intermediate stages of exine diagenesis were more intriguing. The elevated stainability was similar to MULLER's (1959) descriptions for some fossil pollen and seemed in support of FRITSCHE's (1834, 1837) conclusion that exines are not composed of just one compound but several unrelated substances. FRITSCHE specified that one of these was a wax or lipoidal substance. STRASBURGER (1889) and FISCHER (1890) came to somewhat the same conclusion when they concluded that sugars were responsible for the the reddish coloration observed when exines were exposed to concentrated sulfuric acid. FISCHER, who, like FRITSCHE was a chemist, considered that his observations with iodine and aniline dyes indicated the presence of protein in exines.

Thereafter, JOHN's (1814) view that exines were composed entirely of pollenin (sporopollenin) has become widely accepted, in part because of the conclusions of ZETZSCHE and his collaborators (e.g. ZETZSCHE, 1932), although largely, no doubt, because exines of spores and pollen grains from sediments of great age where it is unreasonable to expect the preservation of sugars or protein were still in some cases stainable with basic dyes and became reddish when exposed to sulfuric acid. Hypotheses concerning the nature of sporopollenin have, however, been varied, e.g. that it is a polymer composed of units having the same carbon/hydrogen ratio as that of a terpene (ZETZSCHE, 1932), a highly cross-linked lipid (FREY-WYSSLING, 1953), a high molecular weight polysaccharide (TRAVERSE, 1968), or an oxidative co-polymer of carotenoids and carotenoid esters bound together into a matrix (BROOKS & SHAW, 1968).

Results of extractions which apparently do not alter sporopollenin indicate that there are lipoidal substances either in the interstices of the sporopollenin matrix (TRAVERSE, 1968) or at or near the exine surface (SOUTHWORTH & BRANTON, 1971). Cytochemical testing of intact pollen or exine sections suggest the presence of extractable lipids, protein, and polysaccharide within the substance of the exine. For example, in summary of an extensive study of the cytochemical reactivity of pollen walls, SOUTHWORTH (1973) states that there is lipid, protein (ectexine only), and a trace of polysaccharide in association with sporopollenin.

A method for the extraction of nonsporopollenous substances within the exine was made available by BAILEY's (1960) observation that some exines are soluble in organic bases. Because organic bases are favourable solvents for the extraction of polysaccharides intact (BOUVENG, 1963), our primary effort was given to the possible extraction of polysaccharides from exines. Polysaccharides, expecially acidic polysaccharides, would produce the strong basophilia described for exines or exinous zones of some fossil pollen (e.g. MULLER, 1959; WILSON, 1964; STANLEY, 1966; LEFFINGWELL, LARSON & VALENCIA, 1970) and the strong and stable metachromasia in toluidine blue we observed in some treated exines.

The data reviewed by DEGANS (1967) accents a primary problem in attributing any portion of exine staining in fossil or experimentally treated pollen to polysaccharides. His review emphasizes that carbohydrates are less stable than other components of organic matter in sediments and are eliminated in the early stages of diagenesis.

An additional interpretive difficulty concerns the stainability of the sporopollenin polymer itself. ZETZSCHE (1932) found that sporopollenin reacts with acetic anhydride forming acetylsporopollenin which apparently is brownish-yellow in colour. Sporopollenin

is considered to be strongly osmophilic, indicating the presence of aliphatic double bonds (cf. SOUTHWORTH, 1973, p. 79 for discussion and references). In the hypothetical structure of sporopollenin worked out by LIBERT (1974) drawing from the studies of BROOKS (1970) and HOLLEYHEAD (1974) on carotenoids and sporopollenin, anionic groups not bound directly into the sporopollenin polymer offer an explanation for exine basophilia. The strong basophilia of the exine during both the early microspore period and diagenesis of the exine is explained in LIBERT's (1974: Ch. 7) model by increased frequency of charged groups and linkages during assembly of the sporopollenin matrix and again during diagenesis.

For reasons outlined above our data can be interpreted only morphologically unless the existence within the exine of nonsporopollenous substances, not extractable until sporopollenin is destroyed, is first demonstrated. It would also be desirable to show that these nonsporopollenous substances when embedded within the exine can withstand treatment severe enough to partly destroy sporopollenin. Results of experiments which contribute to the first of these requirements will be outlined below because they are essential to presentation of our results in the present report and, except for abstracts (ROWLEY, 1973, 1975*a*, 1976), these results are not as yet printed (ROWLEY, in press).

Since fractions from exines dissolved in 2-aminoethanol were examined under ultraviolet and some interpretations are associated with the presence or absence of autofluorescence, it is desirable to comment upon exine autofluorescence under ultraviolet. The reasons for exine fluorescence phenomena are not understood (VAN GIJZEL, 1971). VAN GIJZEL, who has developed microphotometrical methods for the study of exine fluorescence (e.g. VAN GIJZEL, 1961, 1967), relates these fluorescence phenomena only cautiously with sporopollenin. He tends to attribute the large spread in fluorescence colour distribution and changes following treatment and increasing geological time to several fluorescent compounds in the exine and their gradual decomposition. Since sporopollenin is considered to be the most resistant substance in the walls of pollen grains and spores, VAN GIJZEL (1971, p. 668) concludes that all these fluorescence phenomena are probably closely related to changes in sporopollenin composition and/or content.

The degradation products of exines dissolved in 2-amino-ethanol (previously acetolysed at 100°C for 10 min and washed in running water for 24 hr to remove nonexinous materials) are soluble in water and can be separated into two fractions by dialysis in cellophane tubing. The dialyzable fraction autofluoresces under ultraviolet, suggesting within the weighted conclusion of VAN GIJZEL (1971) that it includes break-down products of sporopollenin. The non-dialyzable fraction includes filaments or lamellations, and if the exines are dissolved in 2-aminoethanol at high temperatures (130—140°C) for 18 hr this fraction is not autofluorescent in ultraviolet. If 2-aminoethanol treatment is moderate (30 sec to 3 min at 90°C) then the nondialyzable fraction, containing filaments, does fluoresce. When this fraction is extracted in phenol—water, autofluorescent material is restricted to the interface between phenolic and aqueous phases and in the sediment at the bottom of the tube (both are zones where insoluble residues are expected: WESTOPHAL & JANN, 1965). The filaments extracted from the exine occur mainly in the aqueous phase and are not autofluorescent. Recovery of the filaments in the aqueous phase following extraction in phenol—water indicate that their composition is polysaccharide, mucopolysaccharide, and /or lipopolysaccharide (WESTPHAL & JANN, 1965). Both before and after phenol—water extraction, filaments isolated from dissolved exines give a positive cytochemical test for protein, are osmophilic, and are strongly positive for acidic polysaccharides. (They are stained by phosphostungstic acid in chromic acid, bind ferric iron at low pH, and give a

strong and stable metachromasia in aqueous toluidine blue). Since the recovery of protein and glycoproteins is expected in the phenolic phase following phenol—water extraction and only protein conjugated to polysaccharides as mucopolysaccharide occur in the aqueous phase, these results suggest that the composition of filaments within the exine includes mucopolysaccharides and a lipid polysaccharide complex.

SENGUPTA AND ROWLEY (1974) found that strongly basophilic lamellations could be recovered from spores of *Lycopodium clavatum* which had been heated for 100 hr at 300°C under 1kb pressure. Lamellations were observed within residual exines after 100 hr at 350°C, and when these residual exines were etched with 2-aminoethanol (130°C) stainable lamellations could be seen to protrude from the etched surface in sections. These data do not exclude the possibility that the lamellations are composed of sporopollenin, but their resistance to 2- aminoethanol, stainability (e.g. osmophilia and bright red metachromasia in toluidine blue), and morphological appearance are similar to those of filaments and lamellations recovered in the aqueous phase after phenol—water extraction of the nonfluorescent residue of dissolved exines.

MATERIAL

Fresh pollen of *Epilobium angustifolium* L. and *Malva alcea* L. were obtained from plants in cultivation at the Bergian Garden, Stockholm and of *Nuphar luteum* (L.) SM. from Karlbergsjon, Stockholm. Pollen of *Zauschneria californica* Presl. was taken from herbarium specimens (Cult. Rancho Bot. Garden 1952, Balls 8910, S; *Z. californica* var. *villosa* Jeps., California 1948, H. Pollard s. n., S). *Betula verrucosa* pollen was collected and dried with heated air by AB Cernelle (now Pharmacia Uppsala, Sweden) during the 1964 season.

METHODS

Pollen were isolated from anthers without the use of liquids and mixed with pollen of *Betula* and *Zauschneria*. Aliquots were dusted onto the surface of the first solution in each treatment listed in Table 1 while the solutions were stirred, then shaken vigorously to minimize lumping. The mixtures were then placed under vacuum to reduce entrapped air in the pollen grains, then stirred throughout the heating period by bubbling nitrogen through the solutions. The first six treatments listed in Table 1 were replicated four times (*Zauschneria* only once). Except for No. 12 (Table 1) each reaction was terminated by dumping the 15 ml of the treatment solution into 35 ml of ice cold water, centrifuging, and decanting. The pollen exines were washed twice in 50 ml of warm water (ca 50°C) before the next treatment or fixation.

FIXATION AND EMBEDDING FOR TRANSMISSION ELECTRON MICROSCOPY (TEM)

The exines of the replicated treatments (1-6 in Table 1) were fixed in four different ways. The fixations were as follows:

(1) 1% OsO_4 in 0.1 M phosphate buffer (pH 7) for 24 hrs;
(2) 3% glutaraldehyde (GA) in 0.1 M phosphate buffer (pH 7.2) for 1 hr followed by 1% OsO_4 as in fixation No. 1;
(3) 1% GA in 0.1 M cacodylate-HCl buffer (pH 6.9) for 1 hr;
(4) 1% GA in 0.1 phosphate buffer (pH 7) saturated with barium carbonate.

The residues of treatments 7-12 (Table 1) were divided into 3 aliquots and these exposed to fixations No. 1-3. Dehydration was in an acetone series and embedding in epon-araldite (MOLLENHAUER, 1964, mixture No. 1).

Table 1—Summary of Treatments

Treatment	Abbreviations used in text and figure descriptions
0. Fixation without any pretreatment	
1. Acetolysed 1 minute*	(acetol)
2. Acetol+boiled in 10% KOH 10 min	(acetol-KOH)
3. Acetol+KOH+chlorination** 2 min	(acetol-KOH-Cl)
4. Acetol+Cl+KOH	(acetol-Cl-KOH)
5. KOH+acetol+Cl	(KOH-acetol-Cl)
6. KOH+Cl+acetol	(KOH-Cl-acetol)
7. Cl+acetol+KOH	(Cl-acetol-KOH)
8. Cl+KOH+acetol	(Cl-KOH-acetol)
9. KOH+Cl	(KOH-Cl)
10. KOH+acetol	(KOH-acetol)
11. KOH	(KOH)
12. 20% chromic acid for 1 hr at 20°C followed by washing in water at 90°C	(chromic acid)
13. Acetolysed 10 min	(prolonged acetol)
14. Boiled 1 min or 10 min in water	(water)

*The freshly prepared acetolysis mixture consisting of 1 pt. H_2SO_4 and 9 pts. acetic anhydride (Erdtman, 1960) was placed in a water bath (60°C), the water bath was rapidly brought to 100°C, and after boiling 1 min the reaction was terminated by dumping the acetolysis mixture and pollen remnants into ice cold water. The elapsed time from 60°C was ca 5 min.

**Chlorination mixture: 2 ml acetic acid, 3 drops sodium chlorate, 3 drops HCl.

Observations with ultraviolet were made using a mercury vapour lamp (Osram, HBO-200), BG 38, 4 mm or UG-1, 1.6 mm exciter filters or an EK 2a UV blocking filters, Reichert quartz glass immersion condenser or darkfield condenser and 40×0.65 objective. The exines were mounted in water-free glycerine between a quartz slide and cover glass.

Section Staining for TEM

Sections were examined unstained and after the following section stains: (1) 1% aqueous uranyl acetate (UA) for 15 min at 50°C followed by lead citrate (Pb) for 5 min; (2) sections on gold grids were stained with 0.1% phosphotungstic acid (PTA) in 10% chromic acid (PTA-chrom) as described by Rambourg, Hernandez and Leblond (1969) and (3) 5% PTA in 10% acetone (PTA-acetone) after Benedetti and Bertolini (1963), Marinozzi (1968), and Mayo and Cocking (1969); (4) sections on gold grids were immersed in 0.3% thiocarbohydrazide (TCH) disolved in 20% acetic acid for 30 min, washed in 3 changes of 10% acetic acid and then in deionized water (three changes over 20 min), and immersed in 1% silver proteinate (SP) as described by Thiery (1967) in deionized water for 30 min followed by three washes in deionized water; (5) sections on gold grids were immersed in SP for 30 min and then washed. Information on the binding of bireactive

ligands, such as TCH, with osmium and other metals, is discussed by HANKER, WASSERKRUG AND SELIGMAN (1966) and SELIGMAN, WASSERKRUG AND HANKER (1966). THIERY (1967) and COURTOY AND SIMAR (1974) consider that binding of TCH with SP, aldehydes, and native cations within tissues, as well as controls, are necessary for interpretation of these reactions.

RESULTS

The reactions of exine components to the stains listed below are reported without interpretation since some of the stain reactions may be attributed to groups considered to be associated with the sporopollenin polymer as well as to macromolecules embedded within the exine and extractable substances in the interstices of the exine. Abbreviations used for staining procedures and interpretations currently placed upon positive reactions (cf. references cited in METHODS) are as follows:

UA-Pb

Uranyl acetate followed by lead citrate with or without prior exposure of exines to osmium is a nonspecific staining sequence.

PTA—Chrom

Phosphotungstic acid in chromic acid stains acidic polyanions or acidic polysaccharides. PEASE (1968) determined that PTA in an acid solution at ca pH 1.4 intensely stains acidic polysaccharide. It is an effective stain although less vigorous between pH 1.7—2.1, and, while the stain is delicate, most of its polysaccharide specificity is retained through pH 3.0—3.5.

PTA-Acetone

Phosphotungstic acid in 10% acetone, PTA in weakly acid, neutral, and basic solutions (pH 5.5—8.5) is a general protein stain (PEASE, 1968).

Os-TCH-SP

Osmium followed by thiocarbohydrazide (TCH) followed by silver proteinate (SP). The ligand TCH readily binds to osmium and then SP so that the procedure can be expected to amplify sites of bound osmium. Since both TCH and SP may bind to native ions in the exine, controls are imperative (cf. COURTOY & SIMAR, 1974). Since the contrast in sections treated with TCH-SP or SP without prior osmium staining or with SP after osmium was almost as great as after Os-TCH-SP staining. It is concluded that a portion of the contrast is due to the binding of silver proteinate to charged groups in the exine.

SP

Silver proteinate. It probably is linked to electrostatically charged groups which are disassociated at neutral or slightly alkaline pH.

Toluidine Blue

Negative charges must be closely spaced in order to induce polymerization of the dye and produce metachromasia. According to PEARSE (1961, p. 150) where intercharge distance is ca 1nm there is no metachromasia and the dye is blue or purple; where the intercharge distance is ca 0.5 nm, e.g. pectic acid, there is a weak metachromasia and the dye is light pink; in polysaccharides with intercharge spacing less than 0.4 nm there is a strong and stable metachromasia, the dye is pink or red.

Epilobium

Structure within the exine is stained by PTA-chrom (Fig. 1), PTA-acetone, Os-TCH-SP and—SP following treatment which does not alter exine form, i.e. acetol-KOH, KOH-acetol, KOH-Cl. When stained with UA-Pb, with (Fig. 2) prior osmification, sections of these exines have a fine granular appearance but otherwise look like sections of untreated exines. Without osmium UA-Pb staining frequently darkens structures (filaments) within exines (Fig. 3).

Sections of grains treated more severely, e.g. acetol-KOH-Cl in any combination listed in Table 1, stain as above but show a definite loss of exine substance from the nexine (Figs. 4-7) which is minimal in appearance, although still evident, after UA-Pb section staining (Fig. 5). That part of the exine which is extracted by these reactions and thus missing in sections such as Figs. 5-7 will be called *exine matrix* and the lamellations, fibrils, or filaments that become apparent following extraction of exine matrix will, for convenience, be called *filaments*. The filaments are stained by PTA-chrom (Fig 7) moderately stained by PTA-acetone, and covered by silver granules after reaction with—SP (Fig. 6) or Os-TCH-SP. Filaments are also apparent in the sexine, but only at sites where sexinous elements are thin within the section (Figs. 6, 7). Elsewhere within the sexine, at the level of exine destruction shown, staining of the sexine is extraordinarily intense and structure that was apparent in less severely treated exines, e.g. Fig. 1, is masked by the stain.

Extraction of exine matrix in recent pollen was consistently less when KOH was first (Fig. 4) and greatest when KOH was last (Fig. 7) in the successive treatments with KOH-acetol-Cl. In all cases where extraction of exine matrix was evident, extraction was selective. Filaments were more resistant than exine matrix between filaments and the sexine was more resistant than the nexine. (The nexine is as intensely stainable as the sexine when the level of exine destruction is less than in Figs. 5-8. These stages of destruction are shown in Figs. 1, 4, and 9). In addition the nexine of apertural regions was more resistant to these treatments than the nexine elsewhere (Fig. 8). The proximal portion of the nexine was the first portion of the nexine to show extraction of exine matrix in nonapertural regions (Fig. 4) but in apertural regions the distal portion of the nexine is first to be selectively destroyed (Figs. 1 and 9). In Fig. 9 the distal surface of the nexine is etched and the sexine is entirely gone. Since the sexine was, in rare instances, found to be separated from the nexine following treatment with hot water, it can be assumed that this separation is a physical phenomenon. Isolated sexines and nexines were observed after all of the treatments in Table 1.

As the exine matrix was progressively extracted from exines of *Epilobium*, first the nexine became increasingly basophilic, followed by a reduction in nexine stainability; then the sexine became strongly basophilic. With further treatment all staining was negative. At that level of destruction (alteration of staining properties of the filaments) many exine remnants were greatly expanded and separation of sexine and nexine was common but they were, with a known species under consideration, recognizable morphologically. The diameter of exines does not increase much until destruction is great enough to result in stain extinction. The exine remnants were unstained by basic dyes and difficult to see unless phase or interference contrast microscopy was used. These exine remnants were autofluorescent in ultraviolet.

Zauschneria

The diameters of microchannels remain relatively constant as filaments become apparent, indicating that exposure of filaments is due to extraction of exine matrix rather than

simply the result of expansion of exine (Figs. 14 and 15). In the nexine of *Z. californica* both microchannels and irregular channels occur throughout the nexine (Fig. 10) whereas in the nexine of mature *Epilobium* pollen exines, microchannels are common only in the apertural regions (Fig. 8) and the retention of irregular channels is rare. Microchannels of *Zauschneria* are conically enlarged where they contact an irregular channel but elsewhere in the nexine their diameter is similar to microchannels in the sexine (Fig. 10). Filaments were exposed in the nexine and the nexine was substantially expanded (Fig. 13) or missing (Fig. 11) after treatment with acetol-KOH-Cl in any order, but filaments were apparent in the sexine only when KOH was the final reaction (Fig. 14). Even when KOH was the final reaction, destruction of the nexine was non-uniform. Unlike *Epilobium* in which there was consistently less extraction of exine matrix from the nexine subtending apertures, in *Zauschneria* resistant regions were relatively small as seen in sections, irregular in shape and located throughout the nexine (Figs. 13, 15).

The reaction to stains was similar to *Epilobium*. PTA-chrom staining is shown in Figs. 14-15, Os-TCH-SP in Fig. 12, and SP in Fig. 11. The nexine is largely disintegrated before sexine staining is extinguished. The exine remnant (sexine) is autofluorescent in ultraviolet after staining is extinguished.

Malva

Following treatment a peripheral zone was darkened less by staining for protein than were the central portions of bacules, nexine, etc. (Fig. 16). When the exines of mildly treated pollen grains were exposed to osmium a subperipheral zone of low staining was commonly observed in spines and the nexine, whereas spinules, tectum, most bacules, and the periphery of the nexine, like central regions of spines and nexine, were darkly stained. This complex pattern of staining by osmium was intensified although not modified by UA-Pb staining (Fig. 21).

Stainable substances are extracted from the exines of pollen during treatment by acetolysis or KOH (Figs. 16, 21). Exine matrix is apparently not extracted by these treatments since unstained sections have a uniform density to electrons, suggesting that the mass of the exine was relatively unaffected.

Exine matrix was extracted by acetol-KOH-Cl treatment in any order (Figs. 17, 18, 20). The fine structure of the sexine and a distal portion of the nexine were characterized by numerous voids which were unstained by PTA-acetone, PTA+chrom (Fig. 18) and UA-Pb (Figs. 17, 20). Unstained sections of exine showed a reduced density to electrons in the sexine and a distal zone of the nexine (Fig. 19), indicating that the mass of the exine had been reduced in these regions. When KOH was the first treatment followed by acetolysis and chlorination in either order, less exine matrix was extracted (Fig. 17) than when KOH was the final treatment (Figs. 18-20), although the pattern of extraction was similar. When KOH was the final reaction, tips of spines were expanded (Fig. 20) and tended to be unstained by basic dyes. Spines were frequently entirely missing even after treatment in hot water and this phenomenon was attributed to mechanical abrasion.

Following any series of reactions that removed exine matrix, the nexine—and in some cases the spines, stained with exceptional intensity in basic dyes and were pink to bright red in toluidine blue. In sections for TEM it was common to find that the most darkly stained portion of the exine lay between severely and moderately damaged portions of the exine (Fig. 18, see also Fig. 1, and the bases of spines in Fig. 29).

Longer treatment periods than those listed in Table 1 extinguished the stainability

of spines but not of the nexine. Both spines (some greatly enlarged) and nexine were autofluorescent in such cases.

Betula

Exines of *B. verrucosa* are lightly stained by PTA-chrom, PTA-acetone, Os-TCH-SP, and —SP during the late period of pollen development (Fig. 24) and in mature pollen. Following acetolysis (Fig. 22), KOH, Cl, or hot water (Fig. 26) treatment a peripheral zone of the exine may be darkened by these stains. Untreated mature exines are fairly darkly stained by UA-Pb with (Fig. 25) or without prior osmium staining, whereas after the above treatment staining with Os-UA-Pb is greatly reduced and often similar to the light part of the tectum in Fig. 26. A peripheral zone may be stained more intensely, however, (Fig. 22) and structure may be seen within all or portions of the exine (Fig. 23).

All of the three-step treatments listed in Table 1 and chromic acid removed distal portions of the tectum and foot layer. The exine remnant in Fig. 27 shows features typical of these treatments, i.e. the distal part of the tectum is etched or entirely destroyed, the tectum tends to be separated from the nexine resulting in straightening of bacules (which are compressed and offset during pollen maturation), and apertural regions are the least damaged portions of the exine. The delicate and fragile appearing lamellae and associated filaments of the apertural endexine are the last part of the exine to be destroyed (compare Figs. 25 and 27). The proximal surface of the foot layer was never seen to be etched, perhaps because of the thin coating of endexine. After more severe treatment than was given the exine in Fig. 27, e.g. acetol-Cl-KOH or chromic acid, exine remnants increase in diameter and, except for the endexine of the apertures, are unstained by basic dyes or stains for TEM. In toluidine blue the apertural regions of such remnants are pink to bright red and the nonapertural regions are essentially invisible under full cone illumination, whereas exines from the pellet used in the preparation of Fig. 27 were pink in nonapertural regions and the apertures were bright red in toluidine blue. All exine remnants were autofluorescent under ultraviolet.

Nuphar

Results of treatment with chromic acid were similar to successive treatment with acetol-Cl-KOH. The endexine was destroyed, except under spines, spinules tended to be missing or unstainable, and the tips of spines were either missing or weakly stainable (Fig. 29). A section from an untreated pollen grain is shown in Fig. 28. In *Nuphar* the aperture margin was destroyed (left portion of Fig. 29) or lost its stainability before nonapertural parts of the exine, although only the bases of spines were resistant to severe treatment.

In basic dyes it was common to see treated exines with brightly stained spine bases separated by unstained exine. Both spine bases and unstainable portions of the exine were autofluorescent.

DISCUSSION

Exine Stainability

In exines of fresh or air dried pollen there are three possible explanations for exine staining:

(1) Substances within the exine extractible without morphological destruction of the exine, which are assumed to be located within the interstices of the sporopollenin matrix. It is likely that some of these substances are native to the exine but

others, like exogenous protein and inorganic tracers (ROWLEY, 1975b, 1976; ROWLEY & FLYNN, 1971) may diffuse into the exine during periods of natural or experimental hydration. The availability of these substances for staining appears to be highly dependent upon pretreatment, e.g. fixation. Acidic polyanions, possibly acidic polysaccharides, may be stained in exines after fixation that stabilizes carbohydrates but not following exposure to aqueous solutions, including aldehyde fixatives without added cations (ROWLEY & NILSSON, 1972; ROWLEY & DAHL, in press).

(2) Exine stainability has, in general, been attributed to sporopollenin (cf. brief review in INTRODUCTION), except during pollen development or following procedures commensurate with the preservation or specific extraction of substances presumed either to be in some way associated with sporopollenin or transitory within the exine.

(3) Macromolecules (filaments, fibrils, or lamellations) embedded within the exine, apparently unextractable without a morphological alteration of the exine. We assume that these macromolecules are not directly stainable in intact exines because if they were available to stains, they would also be subject to extraction or destruction by the relatively strong reactions that have become a hallmark for sporopollenin and are commonly used in palynological studies. On the other hand, since very severely treated exines, while still autofluorescent under ultraviolet are no longer reactive to stains, it is tempting to wonder if a portion of the stain reactivity in the intact exine, e.g. weak staining in basic dyes, may be produced by the anionic groups of macromolecules encapsulated (and protected) by sporopollenin. Stainable macromolecules embedded within sporopollenin ought to be exposed by sectioning for TEM, and they are (ROWLEY, in press; Fig. 5), although in most sectioned exines their stainability is disconcertingly poor, especially when compared with the stainability of filaments, partly or wholly isolated from exines (ROWLEY, in press; Figs. 11-13). The simplest explanation, drawing upon the apparent elastic as well as plastic nature of the exine (BANERJEE, ROWLEY & ALLESSIO, 1965) may be that exposed surface is largely sealed off either as a result of the heat and deformation produced by the cutting process or the elastic recovery of sporopollenin.

It is necessary to assume that these three possible kinds of stain reactivity within the exines interact competitively or additively rather than exist in isolation. It is probably unnecessary to note that simple explanations for exine staining are likely to be fatuous; they can, however, provide a starting point for dialogue. Thus, in our discussion we will utilize the interpretations, now current, for positive reaction of the stain used (cf. first portion of RESULTS).

RELATIVELY MILD TREATMENT OF EXINES

Our results for exines treated with hot water, the acetolysis mixture, KOH, or chlorine producing solutions emphasize what every palynologist who has used TEM as a tool will know quite well and that is that exine staining is variable. In agreement with all prior reports, whether by chemists, morphologists, etc., the major variables are between pollen of different taxa. In order to avoid comparisons between the stainability of exines before and after treatment and pollen of different taxa we have selected examples of migration of stainable substances where stain disjunctions occur within the ectexine of individual exines.

After treatment in hot water (rarely after acetolysis or KOH and not observed after chlorination) there was a dark zone at the periphery of the exine in pollen of *Betula* stained for protein (Fig. 26) or by UA-Pb (with or without prior exposure to osmium). The zone was not apparent when osmium tetroxide was the only stain. A darkly stainable peripheral zone is a fairly common phenomenon in exines following chemical fixation, especially by aldehydes, e.g. SOUTHWORTH AND BRANTON, 1971: Fig. 11- stained with permanganate; ROWLEY AND ERDTMAN, 1967: Fig. 6. ERDTMAN (1963) suggested *Stegine* as a term for this layer. Our hypothetical explanation for this stegine effect is that a barrier may be affected at or near the surface of the exine by treatment, such as chemical fixation and hot water, which cross links or denatures otherwise extractable protein.

It was only in pollen of *Betula* that we observed a definite stegine, and in *Malva* stainability of the exine margin was low rather than elevated following the above treatments when sections were stained for protein or by UA-Pb without prior exposure to osmium. Staining with osmium resulted in a complex pattern of contrast in which the periphery of spines and nexine (except the proximal surface) were dark while a sub-peripheral zone was light. These results are consistent with an interpretation involving independent migration of two nonstructural substances, presumedly protein and an unsaturated lipoidal compound. They could also be explained by reactions with aliphatic double bonds (osmium) and anionic groups (PTA in the pH range 5.5—8.0) included in model structures of sporopollenin (e.g. LIBERT, 1974).

Phosphotungstic acid at low pH (1.4, e.g. PTA in 10% chromic acid; to at least pH 1.7) is a vigorous stain for acidic groups, e.g. carboxyl and sulphate groups, which are not considered to occur in sporopollenin (e.g. BROOKS, 1971: p. 380). There would be confidence in assignment of positive staining by PTA in chromic acid to acidic polyanions. Nevertheless, because of the recovery of PTA-chrom stainable filaments from dissolved exines in the aqueous fraction of phenol—water extractions and cognizance of saccharides positive staining will be attributed to acidic polysaccharide or, for simplicity, polysaccharide. When stained for acidic polysaccharides after "mild" treatment the exines of *Betula, Epilobium,* and *Malva* (exines of *Nuphar* and *Zauschneria* were not consistently recovered) gave negative results with two exceptions. Following either brief or prolonged acetolysis, structure within the exine was darkened in *Betula* (Figs. 22, 23) and *Epilobium*. In *Epilobium* (Fig. 3) the stained structure was similar in the sexine and nexine and showed a pattern like the exposed nexinous filaments in Fig. 5. In *Betula*, staining tended to be limited to the distal margin of the tectum, microchannels, and thin endexine. The pattern of polysaccharide staining is accordant with the fibrillar remnant of the exine exposed by severe treatment (Fig. 27). The importance we attach to these instances of positive staining for acidic polysaccharides in acetolysed exines is that the results can neither be adequately explained by acidic polysaccharides, which are readily extractable in water (SZIRMAI, 1962), within the intertices of the exine nor by the composition of sporopollenin.

We may consider that the substance of the exine is unaltered by these mild treatments (with appropriate qualification for differences between taxa) since SITTE (1959) found that the form birefringence of the exine was not diminished by acetolysis or chlorine bleach.

MODESTLY SEVERE TREATMENT

The results of sequential reaction in the acetolysis mixture followed by KOH, KOH-chlorination, or KOH-acetolysis did not produce either stegine effects or the intensely stainable exines of more severe treatment. Exine staining was weak or negative after staining for protein but all the other stains listed in the METHODS produced strong contrasting

comparable with Figs. 1 and 2. The stainability of acidic polysaccharides increased from slight in untreated (Fig. 24) or mildly treated exines (Fig. 22) to heavy following more severe treatment (Fig. 1). Toluidine blue staining was also altered from greenish, which is typical of untreated exines (SOUTHWORTH, 1974; DUCKER & KNOX, 1976) and indicative of regularly arranged anionic sites on a compound of high molecular weight, to a light pink colour in these treated exines. The light pink colour and a tendency for the colour to fade (to greenish or blue) is typical of a weak metachromasia and indicates the presence of macromolecules with an intercharge distance of ca 0.5 nm between anionic groups (PEARSE, 1961: p. 248-251). These observations indicate an increase in acidic polyanionic groups available for interactions with stains. Since such groups are unlikely to be a part of the sporopollenin matrix, we consider that their increase is due to partial exposure of acidic polysaccharide embedded in sporopollenin. The morphology of these treated exines is compatible with this supposition, i.e. the exines appear to be selectively degraded.

SEVERELY TREATED EXINES

Filamentous or fibrillar structure was exposed in exines successively treated by acetolysis, KOH, and chlorination, in any order, or exposed to 20% chromic acid for one hour. Some or all parts of these exines stained with extraordinary intensity although the stainability of portions was either weak or negative. While some portions of these exines were missing or swollen, retention of specific form was adequate for recognition and total dimensions of exines were not greatly changed. These phenomena have been observed in exines from sediments. WILSON (1964: p. 430) wrote, with regard to the intense stainability of fossil exines from portions of some sediments, "It is not uncommon to find in a single palynological preparation of wellcuttings, Pennsylvanian fossils [from the stratigraphic level at which the sample was cut] that are unstained, mixed with Cretaceous fossils [derived by 'cave-in' from levels above] stained bright red, and modern pollen [from the atmosphere at the well head] that are pink." In several reports on the intense stainability of some fossil exines STANLEY has emphasized the differential reactivity of exinous layers (colour plates and references in STANLEY, 1966). STANLEY suggests that general age distinctions (within individual cores) are possible, based upon exine stainability as well as recognition of Pleistocene or younger recycled grains. In the latter, the ectexine tends to be moderately stained by basic dyes and the endexine only slightly stained, as FAEGRI AND IVERSEN (1964: p. 18) and FAEGRI (1956: p. 643) consider typical for recent pollen. In exines from sediments of intermediate age (STANLEY specified Late Mesozoic or Early Tertiary age for the work reported in 1966) both the ectexine and endexine may stain with great intensity whereas only the endexine may be stainable, and this very intensely, in sediments of greater age. In sediments of still greater age neither the ectexine nor endexine may stain.

Filaments, fibrils, and lamellations are reported in studies of fossil exines prepared for TEM at levels seldom encountered in exines of recent pollen grains. In two of the sections of pollen from Eocene sediments shown by EHRLICH AND HALL (1959: Figs. 1, 3) the exine appears to be filamentous. The sections are dark (stained by osmium) where filaments are closely spaced and light where filaments are more loosely associated. In a study of exines of *Wodehouseia* from Upper Cretaceous sediments LEFFINGWELL, LARSON AND VALENCIA (1970) compare thin sections stained with lead citrate for TEM with exines stained with the basic dye aqueous safranin-O. The ectexine in these fossil exines is distinctly fibrillar or filamentous and is lightly stained by lead and the basic dye. The

endexine is compact in appearance and heavily stained by lead citrate and is strongly basophilic. LEFFINGWELL *et al.* consider the common occurrence of uniform or negative stainability of the endexine and ectexine and conclude that, while the conditions favourable for the reactions they recorded or the period of their occurrence in the fossilization process are unknown, the low incidence of differentially stainable residues in the fossil record suggest that favourable conditions may exist within rather narrow limits.

DISINTEGRATION OF THE EXINE

By increasing treatment periods beyond those listed in Table 1 for chromic acid or successive exposure to acetolysis, chlorination, and KOH, especially where KOH is the final treatment, remnants of exines may be obtained which are colourless, are not stainable with basic dyes or osmium, and are often greatly enlarged. The exine remnants are easily recognizable. They are still autofluorescent under ultraviolet. Within the pellet obtained through centrifugation there were masses of small rod-like objects, as seen with phase microscopy, and these autofluoresced in ultraviolet. It seems that some exines had come apart (disintegrated) and the sporopollenin had not been destroyed.

SITTE (1959) determined that form birefringence of the exine is extinguished by treatment with chromic acid. Citation of SITTE's observation under our section concerning exines on the verge of disintegration is arbitrary.

Exines which survive extended severe treatment without becoming swollen are caused to swell and then disintegrate by further treatment, including hot water. Their sensitivity to solvents may be due simply to the enormous surface area of the remnant, e.g. the nexine in Fig. 13, although it is tempting to wonder if the macromolecules of the filaments, fibrils and lamellations may be important in exine integrity. Fossil exines which are no longer stainable tend to be yellow or brown in colour and are resistant to solvents, features which are attributed to exine carbonization, e.g. GUTJAHR (1966). Some exines from sediments are, like our very severely treated exines, damaged by mild treatments. For example, from a peat, R. H. TSCHUDY (personal communication) found exines that began swelling immediately and some disintegrated within a few seconds after the addition of 0.5% NaOH.

EXINE EXPANSION

Because exine morphology is not altered by treatment which partially removed exine matrix (e.g. the microchannels in Figs. 12-14) we consider that the exposure of filaments did not result from expansion of exine. On the other hand when the integrity of the exine matrix is degraded beyond a certain level, further treatment results in swelling which does not seem to be a passive separation of the remaining exinous subunits. For example, when chromic acid is used to destroy exines covered by evaporated carbon in the preparation of single stage carbon-surface replicas, the carbon film was often ruptured due to expansion of the exine (FLYNN & ROWLEY, 1967: p. 235). ISHIDA (1970) shows illustrations of exines swollen by reaction with chromic acid, and illustrations of expanded exines following sodium hypochlorite treatment appear in the report of KARAGYOZOVA AND KARAGYOZOV (1975).

We associate swelling with hydration of the macromolecules embedded within sporopollenin and find that treated grains which enlarge rapidly in water do not do so in acetone, ethanol, water saturated with barium carbonate, or other solutions which stabilize polysaccharides. R. H. TSCHUDY (personal communication) found that in some pollen grains successively treated by chlorination-acetolysis-chlorination exines became as much as 70%

larger in diameter than the average size for the species. Swelling to that extent was most likely to occur, he observed, in grains which had become dark brown or black following acetolysis and were chlorinated to reduce the dark coloration.

When the amount of sporopollenin encapsulating the reactive macromolecules is reduced, they become intensely stainable and acetolysis produces an exceptionally dark coloration. Since it is unlikely that these reactive macromolecules would survive our treatments unless protected by encapsulation in an inert material, and since reactivity is not extinguished until further treatment we conclude that stain reactions are expressed while these labile molecules are still embedded within sporopollenin.

Location of Nonsporopolleninous Macromolecules within the Exine

The filaments or fibrils apparent after sporopollenin is partially removed from exines are likely to consist of a core of reactive macromolecules surrounded by sporopollenin. Morphologically, filaments or fibrils persist, as does specific exine form, although the remnant may be etched or expanded, after basophilia etc. is extinguished, presumably due to the destruction of the reactive macromolecules. The composition of these filaments and fibrils could then be entirely of sporopollenin. Thus, while, for convenience, we have referred to the stainability of filaments our reference ought, we believe, to be to reactive macromolecules embedded within sufficient sporopollenin to provide protection but little enough to account for increased stainability.

Based upon the results of our treatments and published observations of fossil exines, destruction of the exine can be selective in the sense that sporopollenin between filaments is more readily extracted than sporopollenin adjacent to (encapsulating) the reactive macromolecules. With qualification for physical factors such as surface area and volume of exinous parts, destruction is also selective in the sense that some portions of the exines were less affected than others (cf. part 3 of the following section).

THEORETICAL CONSIDERATIONS

1. *Specifically ordered polymerization promoting surfaces*—Atkinson, Gunning and John (1972) in their study of sporopollenin in the cell wall of *Chlorella* and other algae conclude, although with acknowledgement of the very limited evidence so far available, that the presence of sporopollenin in algae is related to the deveopment of trilaminar (white line centered lamellations) wall components as well as to the capacity to synthesize secondary carotenoids They refer to the lamellations on which condensation of sporopollenin or protosporopollenin occurs as *receptor surfaces*.

We consider that receptor surfaces for the ectexine or part of the ectexine are elements of the plasma membrane—glycocalyx on microspores within the callosic special cell wall. Rowley (in press) reviews examples of exine formation on glycocalyx components. Portions of the ectexine seem to form on white line centered lamellations, e.g. the footlayer of *Zea* (Skvarla & Larson, 1966) and of *Endymion-nonscriptus* (Angold, 1967), which apparently form on or adjacent to the plasma membrane (or as Dickinson & Heslop-Harrison, 1968, state, form on lamellations which arise from the plasma membrane). In pollen of taxa having pronounced endexines portions of the ectexine as well as the endexine appear to form on white line centered lamellations, e.g. bacules, footlayer, and endexine in the Labiatae (Nabli, 1975) and nontectal bacules, foot layer, and endexine of *Artemisia* (Rowley & Dahl, in press). It must be noted that parallel lamellations with or without white line centres were not observed in our treated exines although they are common in nexines of *Epilobium* and *Zauschneria* throughout early exine formation. We consider it

possible that the white line centered lamellation phenomenon is transitorily superimposed upon glycocalyx components (filaments) as is suggested by the hypothesis of agglutination by polybasic molecules proposed by SENGUPTA AND ROWLEY (1974; cf. part 4 of this section).

On morphological grounds it would seem that the ectexine of *Epilobium* and *Zauschneria* would have formed on different receptor surfaces than the endexine. For *Epilobium* this is in agreement with studies of ontogeny (e.g. BEER, 1906; ROWLEY, 1975c). It would also appear that the spines of *Nuphar* pollen might have formed on different receptor surfaces than the rest of the exine; it is in agreement with the observations of FLYNN AND VOLLMER (1972) for the ontogeny of the exine.

The receptor surface concept for sporopollenin accumulation did not originate with ATKINSON *et al.* (1972). The idea was expressed by ROWLEY AND SOUTHWORTH (1967) and ECHLIN AND GODWIN (1969) based upon receptor surfaces that are different in morphological appearance, following chemical fixation. Brief reviews and much original data concerning sites of sporopollenin deposition on microspores and tapetal cells are presented by DICKINSON (1976a, b). DICKINSON (1976b, p. 331) refers to sporopollenin receptor surfaces as *ordered polymerisation promoting surfaces*, which should seem to favourably express the concept for exines of pollen grains and spores except for one important point, i.e. specificity of the form of these exines. That point could be incorporated by referring to these macromolecules as specifically ordered polymerization promoting surfaces (SOPPS). The importance of the sporopollenin receptor surface concept as expressed by ATKINSON *et al.* (1972) for *Chlorella* is that in *Chlorella* there are available genotypes which do or do not produce receptor surfaces for assembly of sporopollenin and genotypes which do or do not have the capacity to synthesize secondary carotenoids.

2. *Specificity of macromolecules embedded within exines*—Accumulation of sporopollenin upon plasma membrane glycocalyces offers the possibility theoretically that the composition of SOPPS macromolecules which become embedded within sporopollenin are unique for each taxa. If this is the case, the potential is extraordinary. In most cells and tissues uncontaminated fractions of the labile cell surface macromolecules, e.g. acidic polysaccharide and protein moieties, are difficult to isolate, whereas macromolecules, presumably of cell surface coating origin, embedded within sporopollenin in exines are protected from degradation even during unbelievingly rigorous chemical and physical treatment. Thus sources of contamination can be eliminated and since sporopollenin is dissolved in the organic base 2-aminoethanol from which intact polysaccharides can be recovered, isolation of these SOPPS macromolecules is technically feasible.

There is reason to believe that the study of BOUVENG (1963) on polysaccharides in pollen offers an example of the kind of results obtainable from macromolecules embedded within exines. BOUVENG's aim was to study the polysaccharides in the intine of *Pinus mugo*. Hot monoethanolamine was used in order to make intine polysaccharides more accessible for subsequent extraction through removal of the exine, following BAILEY's (1960) observation that monoethanolamine dissolved the exine (It is likely that the organic base used by BAILEY was 2-aminoethanol, since it dissolves many exines within his stated conditions; the labeling of organic bases by many chemical supply houses remains indefinite). BOUVENG reported that the exine of *P. mugo* appeared to be morphologically intact following treatment with monoethanolamine although, since a considerable amount of high molecular weight polysaccharide was present in the monoethanolamine fraction it was analysed and found

to contain a unique polysaccharide which BOUVENG referred to as a xylogalacturonan. BOUVENG's detailed descriptions leave little doubt that sporopollenin has actually been removed from at least part of the exine during reaction with hot monoethanolamine. He reported that the pollen wall lost only little of its original thickness and consisted of an outer striated and inner nonstriated layer after exposure to monoethanolamine. Subsequent treatment, however, with hot water or with cold 7% NaOH completely removed the outer layer. Since sporopollenin is not dissolved in either hot water or cold hydroxide, it can be concluded that the monoethanolamine did alter the exine, and while the source of the unique polysaccharide is problematical it could have been from within the exine.

If glycocalyx components are embedded within exines and protected by being encapsulated, even bound into the sporopollenin matrix, as specifically ordered polymerization promoting surfaces (SOPPS), then determination of their composition, as we think BOUVENG (1963) made, may be only the tip of the iceberg. Much more could be expected theoretically by way of serological specificity.

3. *Selective destruction of sporopollenin*—Our results indicate that sporopollenin, in certain parts of the exines we have treated, is more resistant to our reactions than sporopollenin elsewhere in these exines, for example the nexine near apertures in *Epilobium*, spine bases in *Nuphar*, and the sexine of *Epilobium* and *Zauschneria*, the endexine of *Betula*, and the proximal portion of the nexine in *Malva*. The outstanding example, however, of selective destruction of sporopollenin is in the filaments and fibrils observable in many of our illustrations. The location of filaments or fibrils revealed after any of the sequential treatments or after chromic acid was consistent within taxa and may be taken as a reflection of SOPPS induced form. Since the patterning remains unchanged after stainability is eliminated and the remnant is still autofluorescent it can be assumed that these filaments or fibrils represent a sporopollenin cast from which SOPPS macromolecules have been destroyed, in the case of nonstainable exines. It would seem that sporopollenin adjacent to or on SOPPS macromolecules is more resistant than sporopollenin located more remote from these molecules. Without knowledge of the reason for the apparent variation in the resistance to sporopollenin, e.g. cross linking in the sporopollenin matrix, it can be suggested that resistant portions of exines are likely to have more closely spaced receptor macromolecules than the less resistant portions of the exine.

4. *Extraction of microfossils from sediments*—Methods for extraction of microfossils from sediments have become sophisticated and relatively efficient. Contemporaneous with HAFSTEN's (1959) observation that loss of pollen exines during chemical treatment could be both precipitous and selective there has been some emphasis upon mechanical rather than chemical methods, at least as a means of monitoring selected parts of geologic sections. Our results suggest that, except for the equivalent of untreated exines or highly carbonized material, further progress could follow adaptation of methods comparable with cytochemical management of polysaccharide, protein, or lipids in tissues. As an example, SENGUPTA AND ROWLEY (1974) suggested that the white line phenomenon in white line centred lamellations, so common in exines of pollen and spores, might be an agglutination effect produced by basic polyelectrolytes bridging the space between adjacent filaments. Regardless of the validity of that hypothesis, we find that filaments isolated from dissolved exines are bound in pairs and larger associations by the addition of polybasic molecules. There is the possibility that strongly

degraded exines, which tend to expand and then disintegrate in aqueous solutions, could be "welded" into physically and chemically strong units by the addition of polybases during the rock grinding process. Bond strengths can be exceedingly great and an absorbed polybase layer is often virtually impossible to remove (KATCHALSKY, 1964). The application of histochemical methods to extractions pertaining to microfossils in rocks may be read as ridiculous although implementation may involve nothing more bizarre than the addition of, for example, high levels of barium carbonate or phosostungstic acid to the acids used during extraction and adjusting the concentration of the acids for the lowest pH consistent with stability of the added ions.

5. *Exine surface coatings*—It would be useful to have a source of sporopollenin in exines from which embedded nonsporopollenin molecules could be extracted by gentle methods. It seems quite possible that exine surface coatings may have a sporopollenin component and in the case of these coatings the protein and polysaccharide portion of the coating is easily removed, in fact special methods are required in order to prevent extraction (PETTITT & JERMY, 1974; ROWLEY, FLYNN, DUNBAR & NILSSON, 1970; ROWLEY, 1971). The reasons for thinking that exine surface coatings include sporopollenin are as follows. LEFFINGWELL *et al.* (1970: p. 242, Pl. 5, Fig. 4) found filamentous strands coating the exine of *Wodehouseia* which, ". . . are obviously resistant to chemical treatment and appear to be of the same composition as the ektexine, only less densely packed". KEDVES, STANLEY AND ROJIK (1974) have demonstrated granules, some with unstained centres, at the surface of exines of *Restioniidites* and *Thomsonipollis* recovered from Lower Eocene sediments. It was their tentative conclusion that the exine had been more-or-less degraded during the sedimentation process and that it is the degraded sporopollenin macromolecules which are visualizable as darkly stained structures at the exine surface. ROWLEY AND DAHL (in press) find that fibrils (which may be SOPPS macromolecules) within the exine of *Artemisia* pollen appear to be continuous with the exine surface coating. In one of the micrographs of KEDVES, *et al.* radially orientated structures within the exine appear to extend out into the region of the exine surface coating of darkly stained granules (KEDVES *et al.*, 1974: Pl. 4, above portion of section labelled "B"). The special methods which have resulted in stabilization of protein and polysaccharides of exine surface coatings are similar to conditions common in ponds and bogs, e.g. relatively high levels of cations such as calcium or ferric iron. Experimentally surface coatings readily bind cations and when heavy metals are involved the coatings are effectively stained for TEM. Exine surface coatings stained with copper and ruthenium compounds and which morphologically appear as short tubules, much like those illustrated by KEDVES *et al.* (1974: Pl. 2; Pl. 5, Fig. 2), are shown by ROWLEY (1971, Figs. 4-5).

Unless the reactive molecules within exine surface coatings are stabilized by metals which are visualizable with the TEM, stains for protein or acidic polysaccharides may be required before the coatings are apparent (cf. ROWLEY & SKVARLA, 1976). Thus, if sporopollenin is as unreactive to stains as our observations indicate, then fine filaments of sporopollenin from which protein and polysaccharide had been lost could escape detection at the exine surface unless special methods such as negative staining were used.

6. *The inertness of sporopollenin*—Following our series of treatments exine remnants are colourless, nonreactive to basic dyes and osmium tetroxide, and do not become reddish in concentrated sulfuric acid. In so far as these remnants, which retain much of their

morphological specificity and continue to be autofluorescent in ultraviolet, are representative of sporopollenin, the above observations indicate that sporopollenin is more inert than we have believed.

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EXPLANATION OF PLATES

PAATE 1

1-3. Sections from the same treatment series. Stained with PTA-chrom (Fig. 1) filamentous structure within the sexine (S) and distal portion of the nexine (arrow) is prominent and the exine matrix appears to be etched (arrow). Stained with osmium-UA-Pb (Fig. 2) the exine appears to be more granular than in untreated exines but the structure within the exine, apparent in Figs. 1 & 3, is obscured. Apparently it is osmium in combination with UA-Pb which obscures structure within the exine since filaments are differentiated by UA-Pb staining without osmium (Fig. 3). Treatment: KOH-acetol; Fixation: Figs. 1 & 2 GA-OsO_4, Fig. 3 GA; Stain: Fig. 1 PTA-chrom, Figs. 2 & 3 UA-Pb. Magnification scale line (Mag. scale): ca. 0.5 μm.

PLATE 2

4. Exine matrix is lost from the proximal portion (between arrowheads) of the nonapertural nexine of *E. angustifolium* earlier than elsewhere in the exine. Treatment: KOH-Cl-acetol; Fixation: GA-OsO_4; Stain: UA-Pb. Mag. scale: 1 μm.

5-6. Exine matrix is extensively removed from the nexine in these sections from the same exine of *E. angustifolium*. More of the residual exine is stained by osmium-UA-Pb (Fig. 5) than by osmium-SP (Fig. 6). There are filaments in both which are essentially unstained (arrows). Where the sections are thin, structure is evident within the sexine (S, arrow heads). Treatment: acetol-KOH-Cl; Fixation: GA-OsO_4; Stain: Fig. 4 UA-Pb, Fig. 5 SP. Mag. scale: 0.5 μm.

PLATE 3

7. When KOH was the final treatment in successive reactions with acetol-Cl-KOH, filaments were exposed in the sexine (S) as well as in the nexine (N). Because of the slenderness of the filaments in the nexine, extreme basiphilia of the exine (especially the sexine), and stickiness of the exine we consider that the exine matrix, which previously enveloped the filaments' is destroyed to the extent of exposing the reactive macromolecules within filaments. Treatment: acetol-Cl-KOH; Fixation: GA+barium carbonate; Stain: PTA-chrom. Mag. scale: ca. 0.5 μm.

PLATE 4

8. This section through an apertural region of *E. augustifolium* pollen grain is from the same grid as Fig. 5. The numbers to the right of the exine indicate the relative positions of Figs. 1-7 with respect to apertures. The exine near apertures, especially the nexine in the region between the arrows was more resistant to treatment than the nonapertural exine. Treatment, fixation, and staining same as for Fig. 5. Mag. scale: ca. 5 μm.

9. The sexine was occasionally entirely separated from the nexine (N) during treatment even with hot water. When nexines were detached from the sexine. the distal surfaces of isolated nexines were etched by successive treatment in acetol-KOH-Cl, in any order. Treatment and fixation same as for Fig. 5; Stain: PTA-chrom. Mag. scale: ca. 5 μm.

PLATE 5

10. In *Zauschneria californica* pollen exine microchannels (arrows) become permanent in the nexine (N) as well as in the sexine (S) and except where they bisect irregular nexinous channels they are the same diameter (cf. Figs. 13-15). Treatment: acetol; Fixation: GA-OsO_4; Stain: UA-Pb. Mag. scale: ca. 1 μm.

11. The fibrils in the nexine of *Zauschneria* are exposed by successive treatment with acetol-KOH-Cl, in any order, although in the sexine only when KOH is the final reaction (cf. Fig. 14). In this figure the nexine (N) is largely destroyed and the remnant is expanded and only weakly stainable. The sexine is fairly normal in appearance although some bacular elements are thin (arrow), probably due to stretching. Treatment: Cl-KOH-acetol; Fixation: GA-OsO_4; Stain: SP. Mag. scale: ca. 1 μm.

12. After acetol or KOH or both treatments, osmium followed by TCH-SP, to amplify sites of bound osmium, produces the pattern of silver granules seen in this figure. Parts of the exine not at the section surface and thus unavailable to TCH and SP show osmium staining without amplification (arrows). Treatment: KOH-acetol; Fixation: OsO_4; Stain: TCH-SP. Mag. scale: ca. 1 μm.

PLATE 6

13. The exine matrix in the nexine (N) was extensively although nonuniformly removed by the treatment, as was typical of *Z. californica* pollen. Microchannels are not enlarged (arrows) by treatment which has destroyed exine matrix. The nexine in the region around the "N" was expanded and in other grains in the section portions of the nexine were missing. The integrity of the sexine (S) is similar in appearance to untreated or acetolysed pollen (cf. Fig. 10). Treatment: KOH-Cl-acetol; Fixation: GA; Stain: UA-Pb. Mag. scale: ca. 1 μm.

PLATE 7

14. The figure includes only a distal portion of the sexine of a *Zauschneria* pollen grain. Destruction of the exine matrix from the sexine exposes a network of fibrils or fine filaments similar to those in the nexine. Treatment: acetol-Cl-KOH; Fixation: GA+barium carbonate; Stain: PTA-chrom. Mag. scale: ca. 0.5 μm.

15. The figure shows a distal portion of the nexine of *Zauschneria*. Destruction of the exine matrix appears to have been greater in the region of the "N" than in the region of the "M". Microchannels (M) are elliptic due to section compression, transverse to the direction of the knife track (dark stripe between "N" and "M"). The extra thickness of the section along the knife track is useful since that part of the section is effectively printed darker than elsewhere, revealing background granulation. The background granulation is similar in the bacular arcade (arrow) and in the microchannel (M), where no exine matrix would be expected, and in the nexine (N) where exine matrix would be expected. Thus, we conclude that little exine matrix remains between nexinous filaments. Treatment, fixation, staining, and mag. scale same as Fig. 14.

PLATE 8

16. Stainability of a peripheral zone of the exine of *Malva alcea* pollen is reduced by acetolysis. The depth of this zone is marked by the penetration of the heads of arrows into the spine (S), tectum (T), a bacule (B), and the nexine (N). Since the section is stained with PTA-acetone the pattern of staining suggests denaturation or extraction of proteins from the surface zone of the exine. Darkly stained material occurs as globules (arrow heads) within the nexine. Treatment: acetol; Fixation: GA; Stain: PTA-acetone. Mag. scale: ca. 1 μm.

17. The exine matrix in pollen of *Malva* is partly removed from the tectum (T) and its spinules (arrow head), bacules (B), spines (S). and the distal portion of the nexine (N). Filaments can be seen where connections between exinous processes are thin (arrows). Treatment: KOH-Cl-acetol; Fixation: GA-OsO_4; Stain: UA-Pb. Mag. scale: ca. 1 μm.

PLATE 9

18. The exines of untreated mature pollen grains of *M. alcea* are essentially unstained by PTA-chrom so that the dark stain in this section can be taken to indicate that acidic polyanions are exposed by removal of exine matrix from spines (S), tectum (T), bacules (B), and a distal portion of the nexine (between "N" and the arrow). Staining is heaviest where destruction of exine matrix is moderate (region of the arrow) and lightest where destruction is greatest (S and T). Treatment: acetol-Cl-KOH; Fixation: GA; Stain: PTA-chrom. Mag. scale: ca. 1 μm.

PLATE 10

19. Section of *Malva* from the same exine as Fig. 18 although without any stain. The density of the sexine (S) and a distal zone of the nexine (N) to electrons is less than for the middle and basal portion of the nexine indicating that high molecular weight substance, presumably sporopollenin, has been lost from the former. The fine radial striations are knife tracts. Treatment: acetol-Cl-KOH; Fixation: GA; Stain: none; TEM: micrograph taken using a 25 μm objective aperture for increased contrast (otherwise a 50 μm aperture was used) and overexposed to increase contrast; Printing: overexposed on hard paper to increase contrast; Controls: The density to electrons is the same throughout untreated and acetolysed exines. Mag. scale: 0.5 μm.

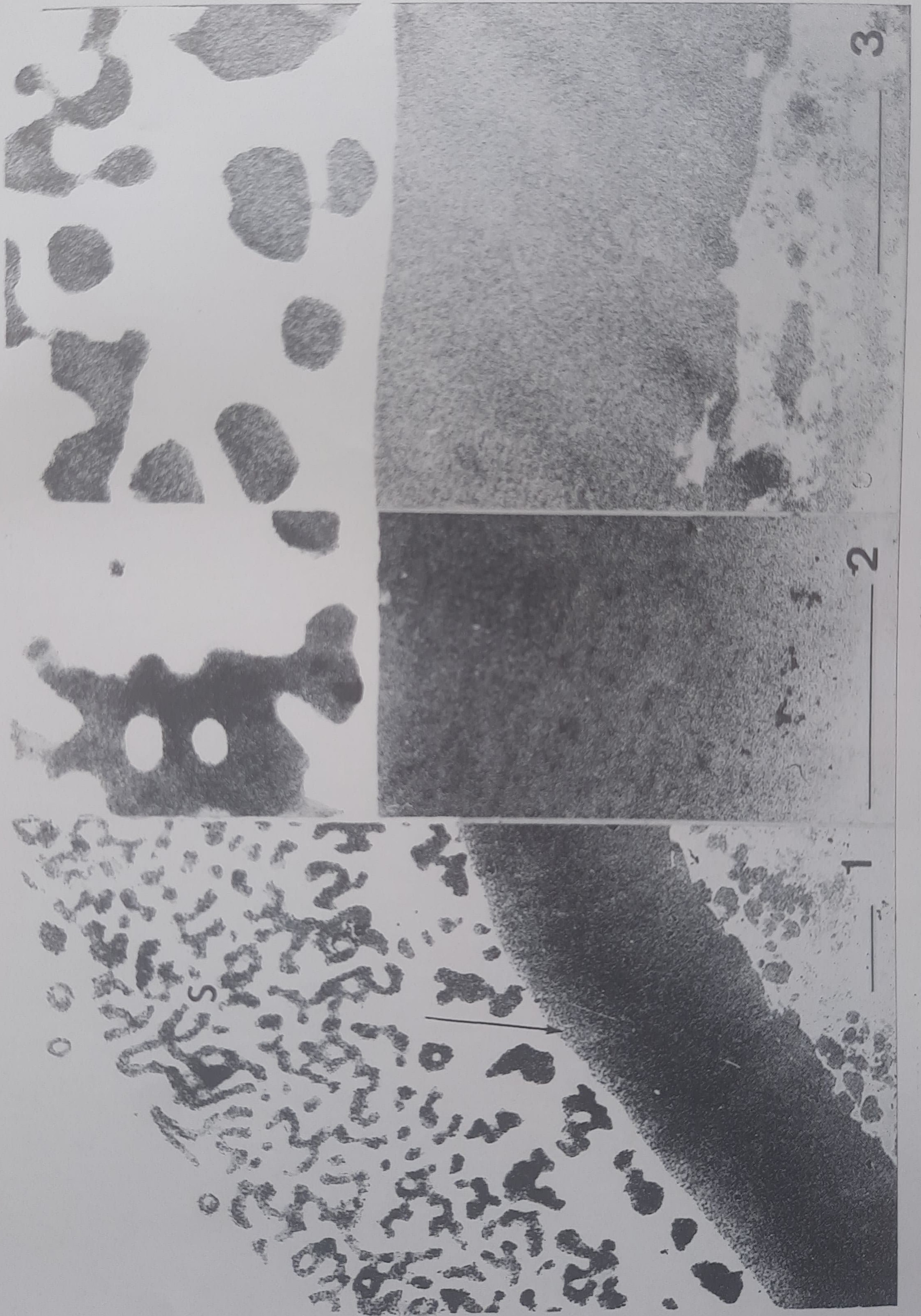
PLATE 11

20. Section from a *Malva* pollen grain treated with the grain in Figs. 18 and 19 through GA fixation after which this exine was exposed to OsO_4 and section stained with UA-Pb. At the tip of the medially cut spine the exine matrix has been destroyed to the extent that filaments have become dispersed; elsewhere the exine appears more compact than in Fig. 18, possibly due to staining with osmium-UA-Pb. Treatment: acetol-Cl-KOH; Fixation: GA-OsO_4; Stain: UA-Pb. Mag. scale: ca. 0.5 μm.

21. Section of an aperture in an exine of *Malva* pollen treated with KOH. The zone of reduced contrast at the periphery of the exine is considered to be the result of extraction of nonstructural substance from the exine. Treatment: KOH; Fixation: GA-OsO_4; Stain: UA-Pb. Mag. Scale: ca. 1 μm.

PLATE 12

22. Following acetolysis the exine of *Betula* is in general lightly stained although a peripheral zone (arrow heads), the microchannels (short arrows), and the thin endexine (E) are stained more darkly. The darkened peripheral zone may extend through the tectum (T) and into the nexine (long arrows). Treatment: acetol; Fixation: GA+barium carbonate; Stain: PTA-chrom. Mag. scale: ca. 1 μm.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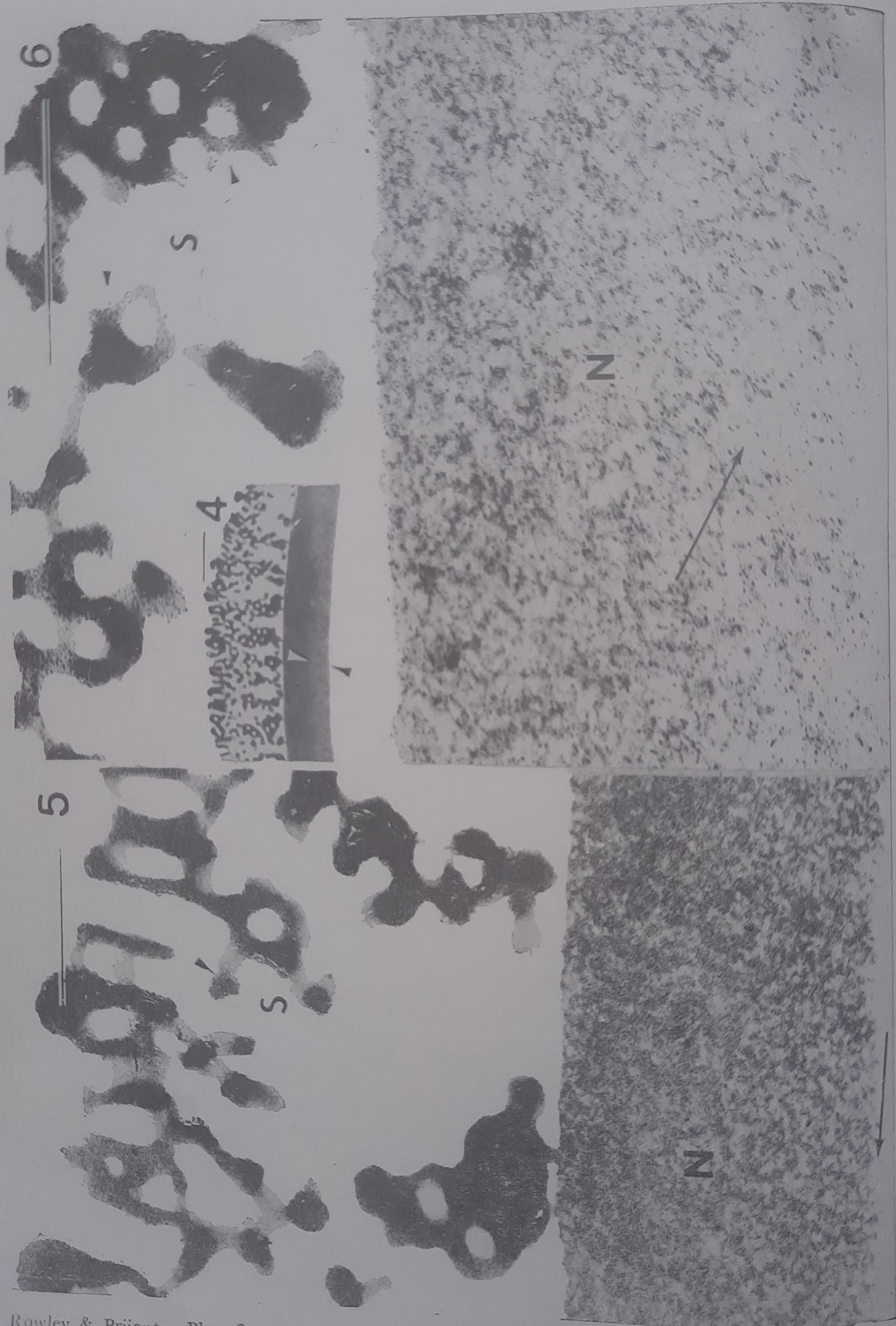
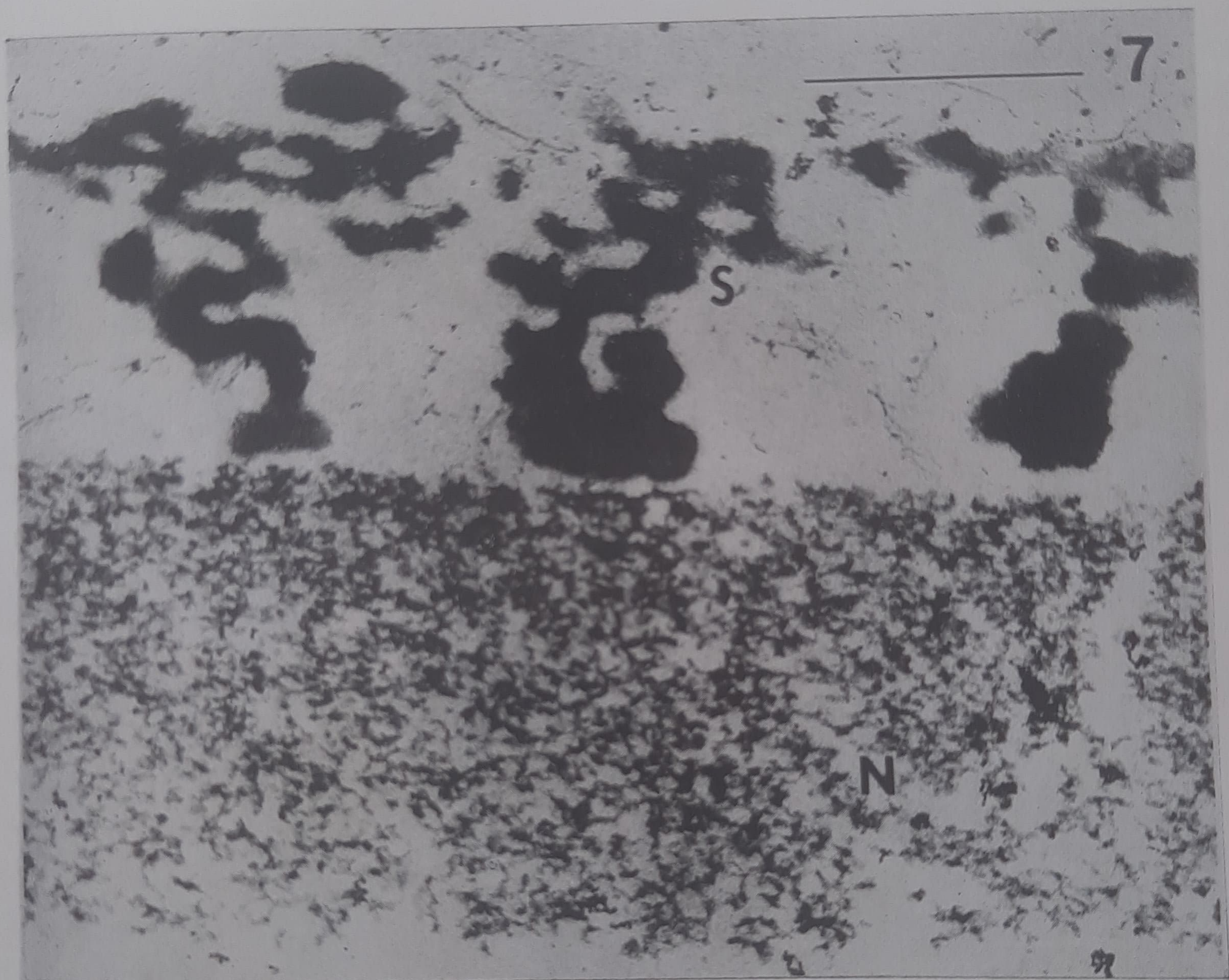


Rowley & Prijanto—Plate 2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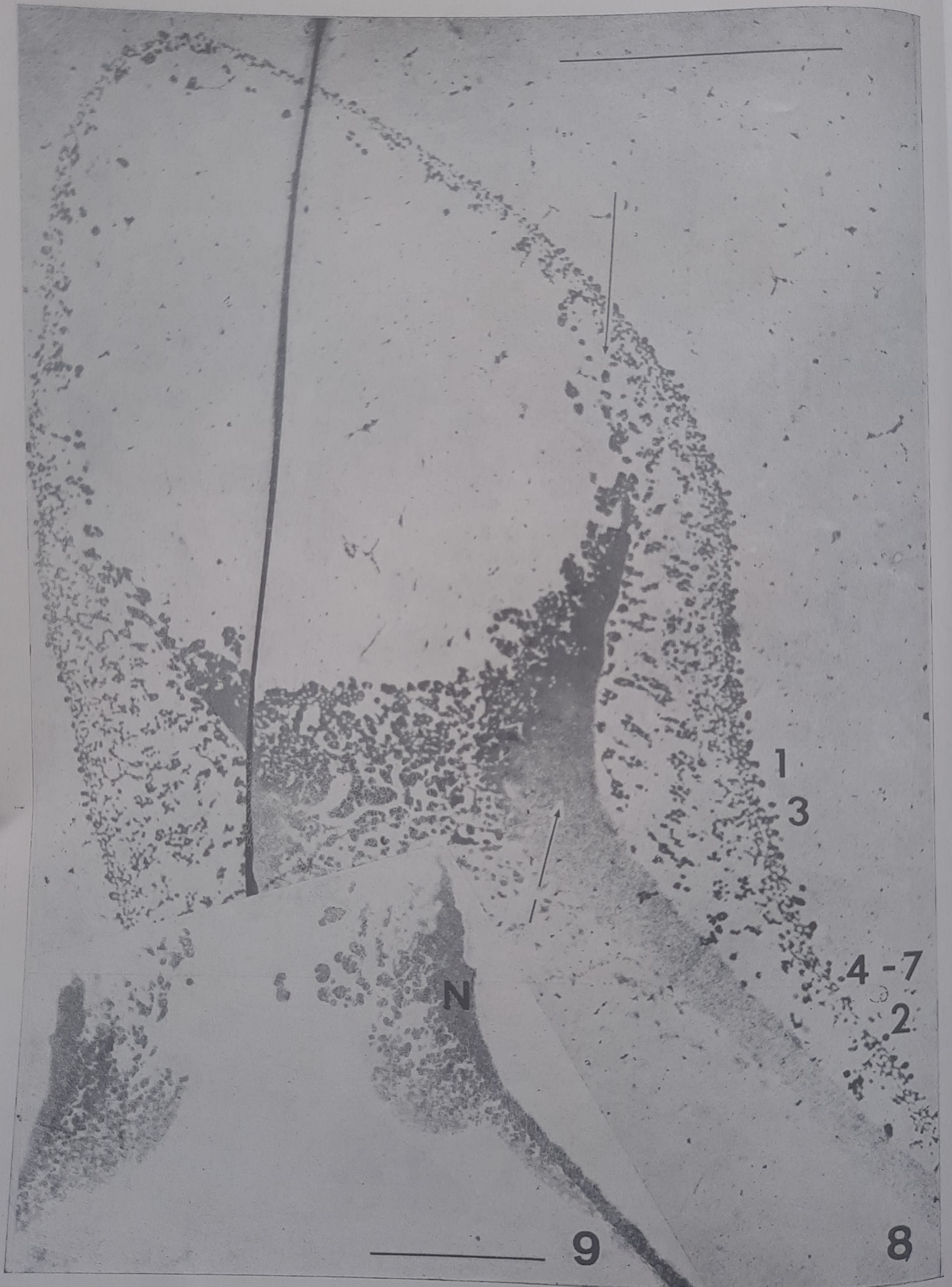
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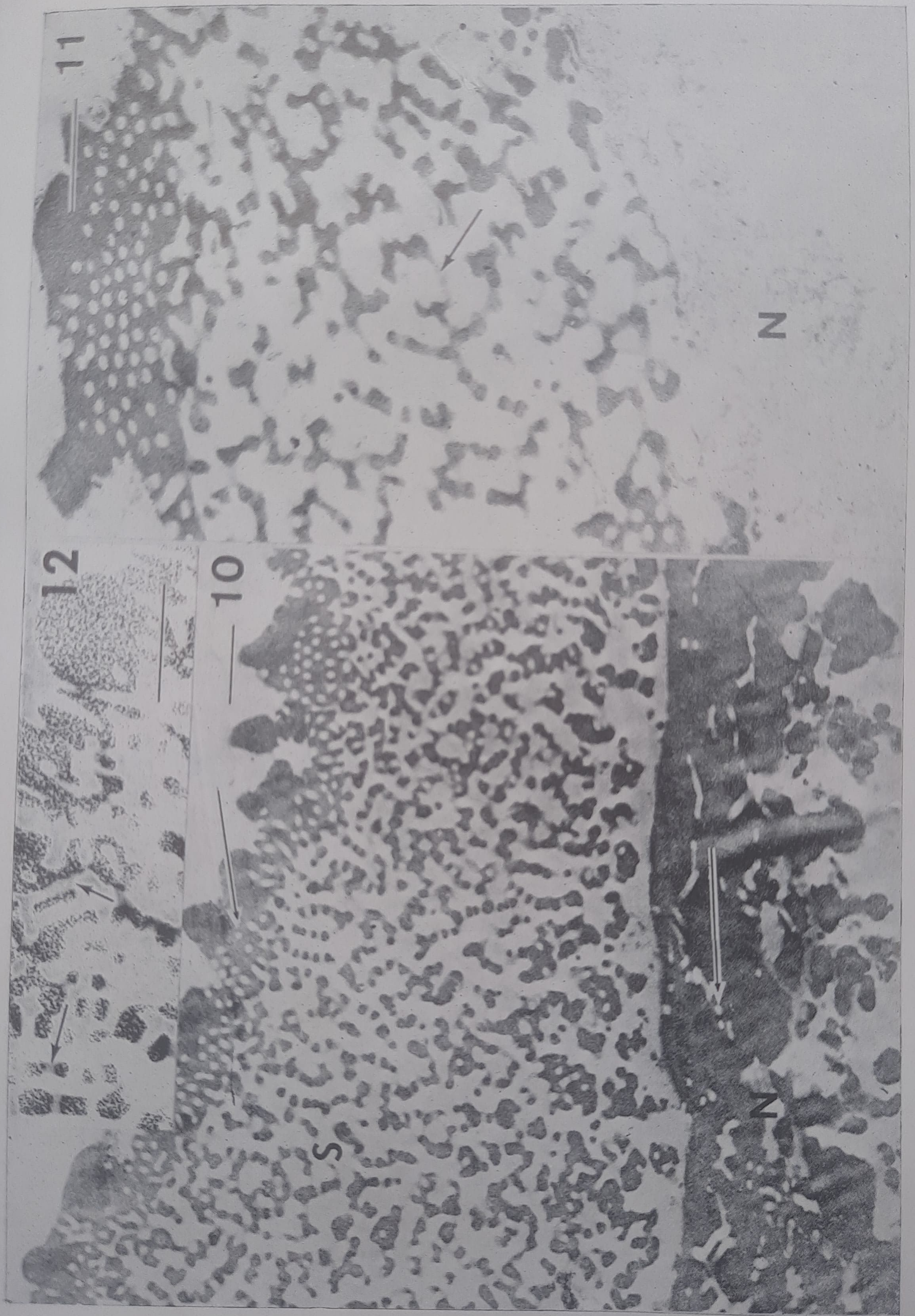
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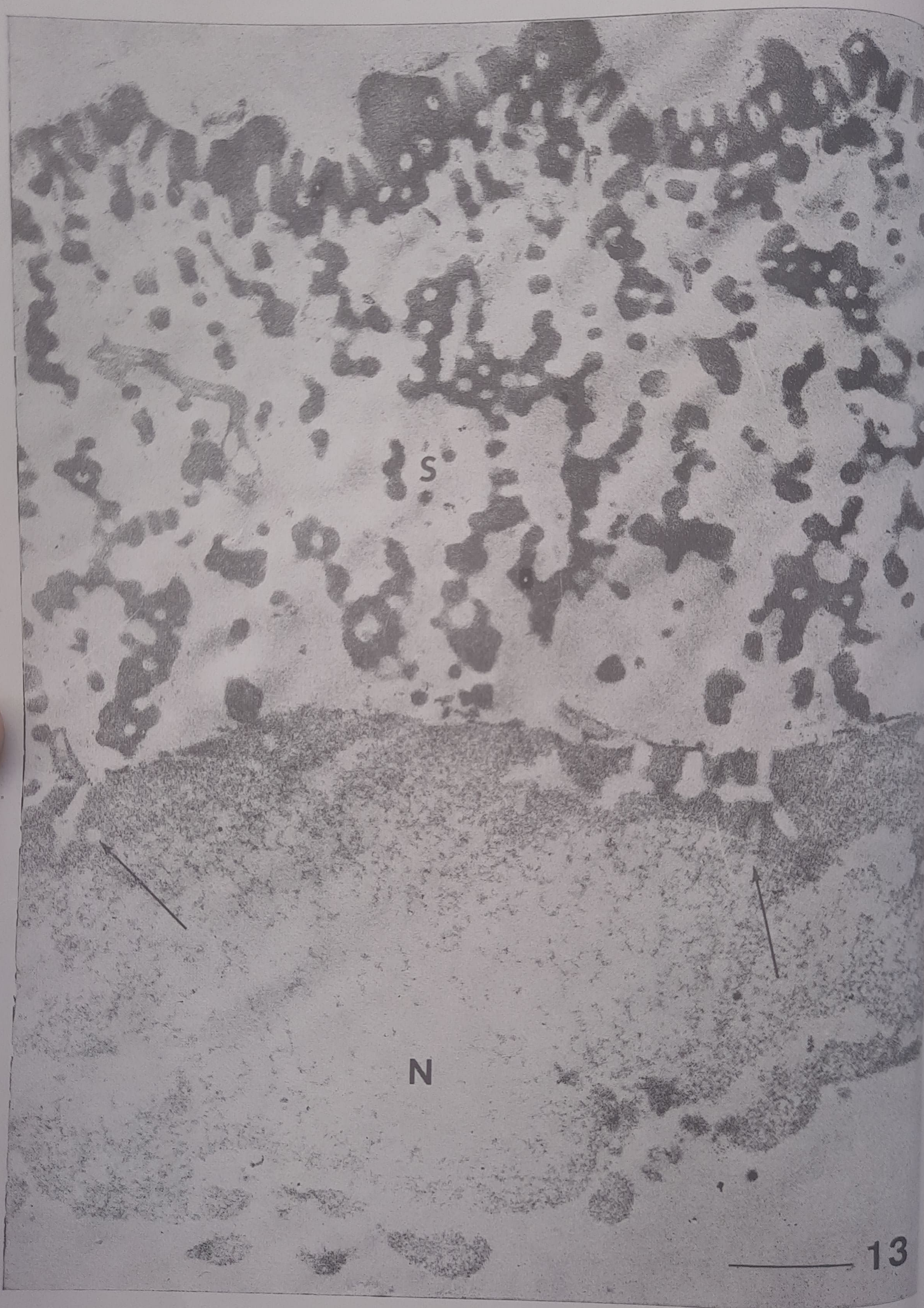
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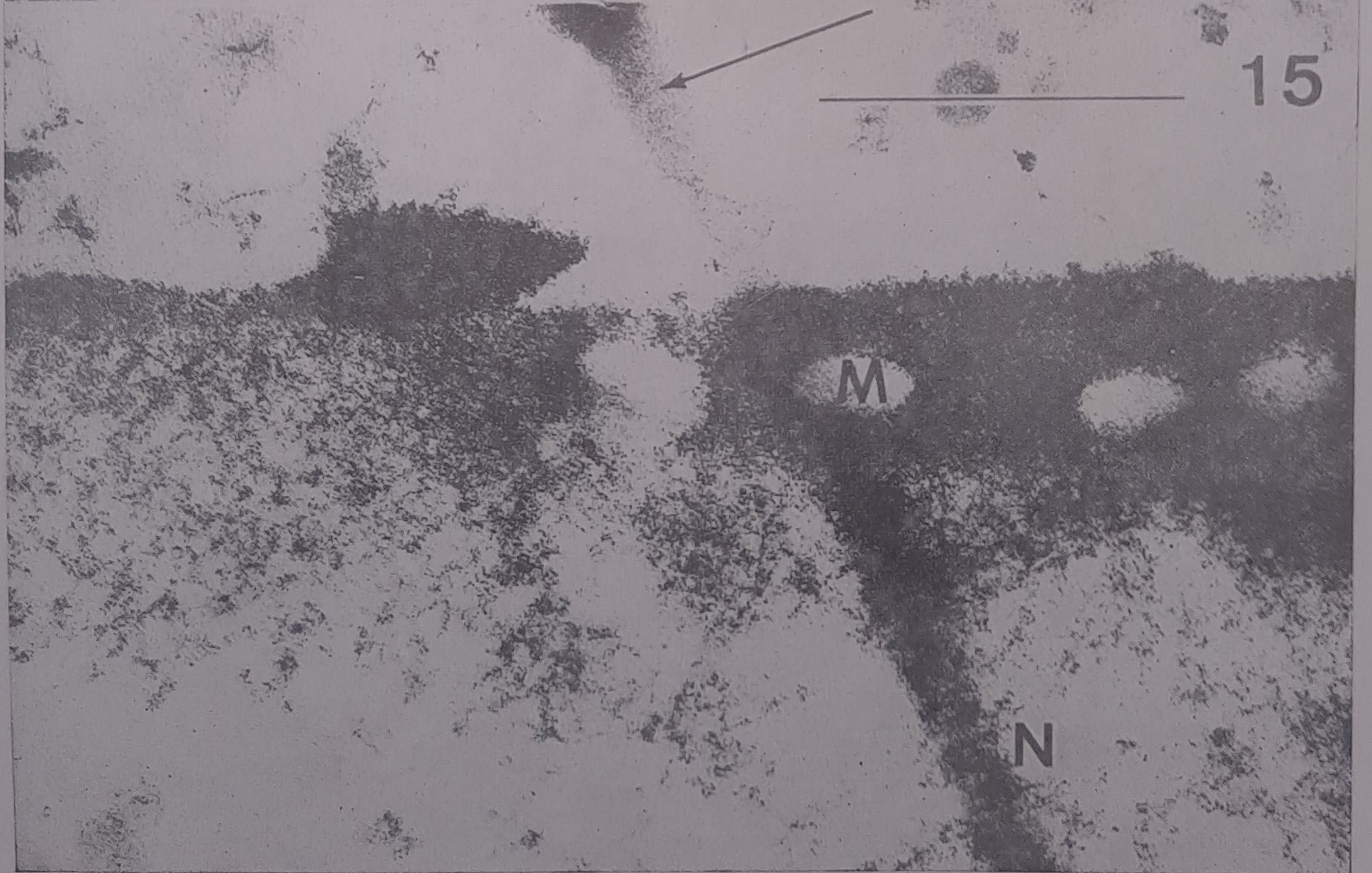

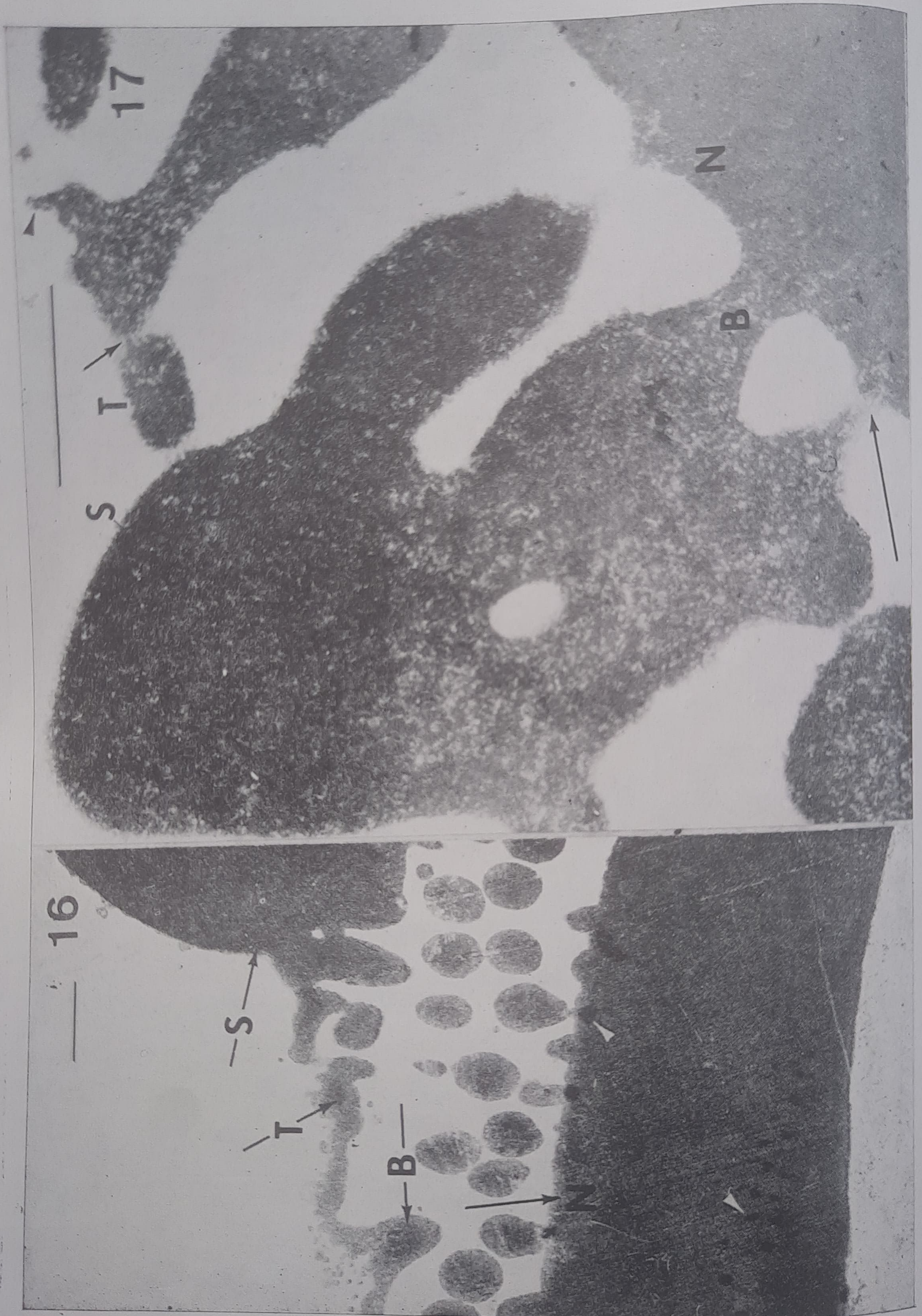
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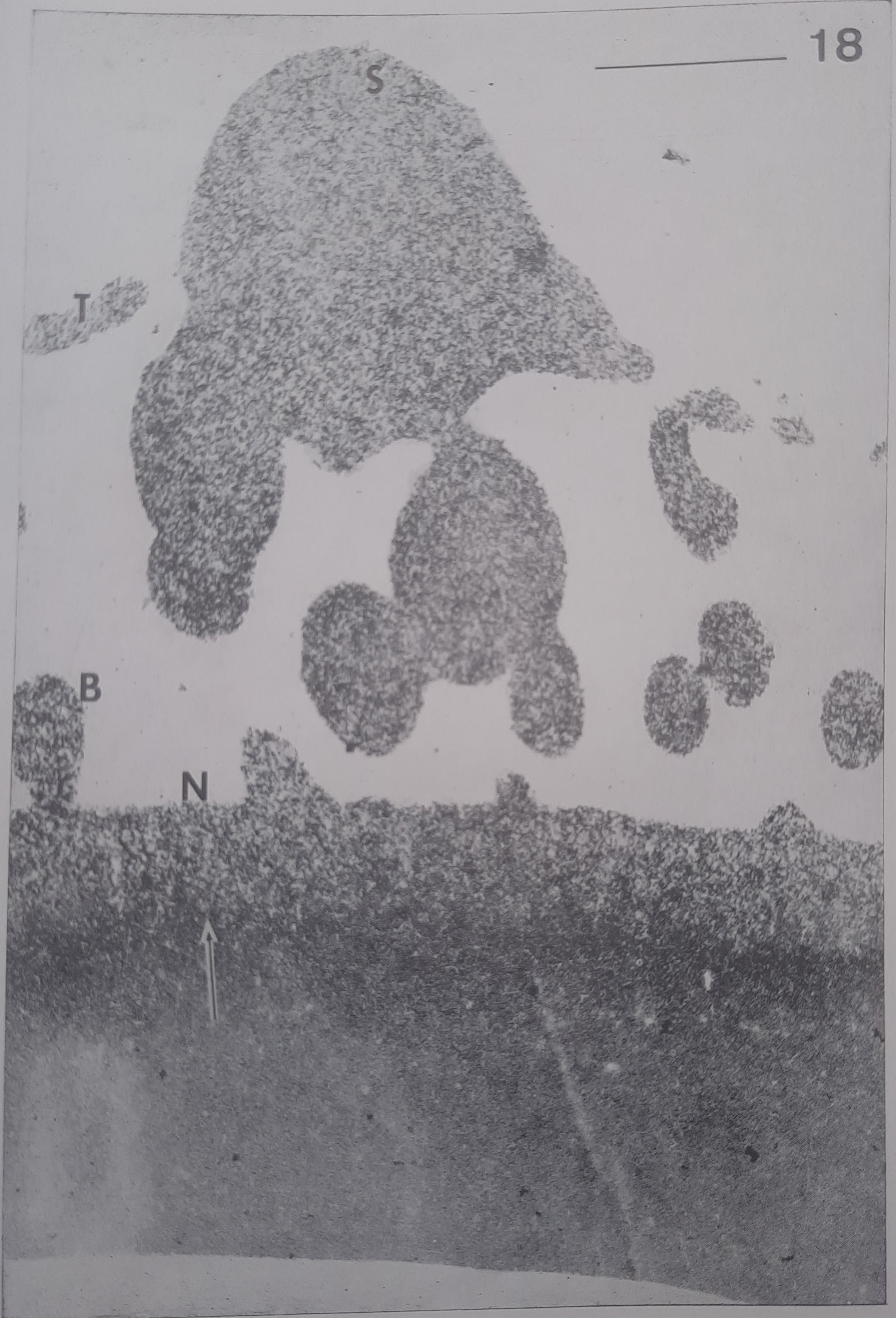

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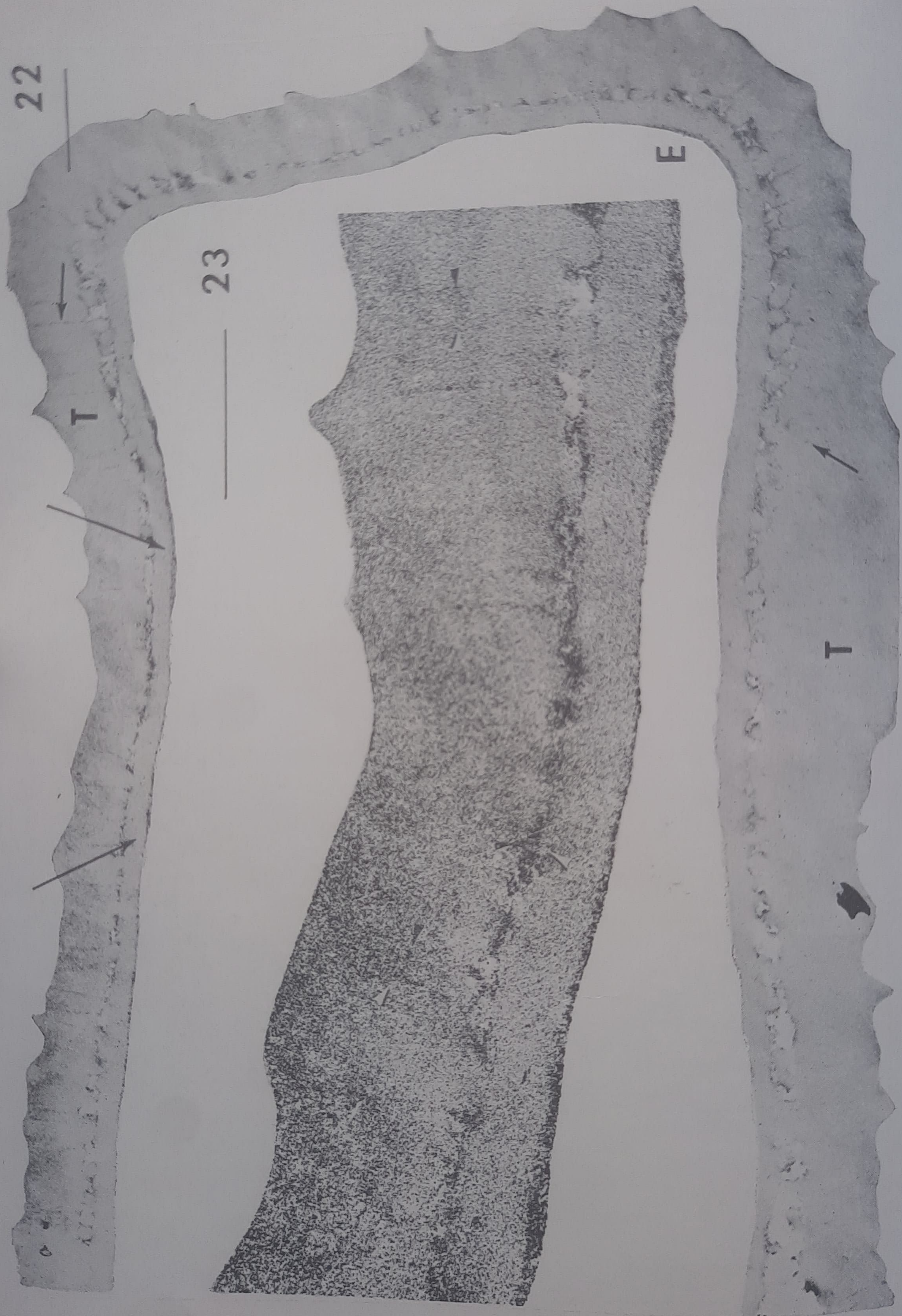
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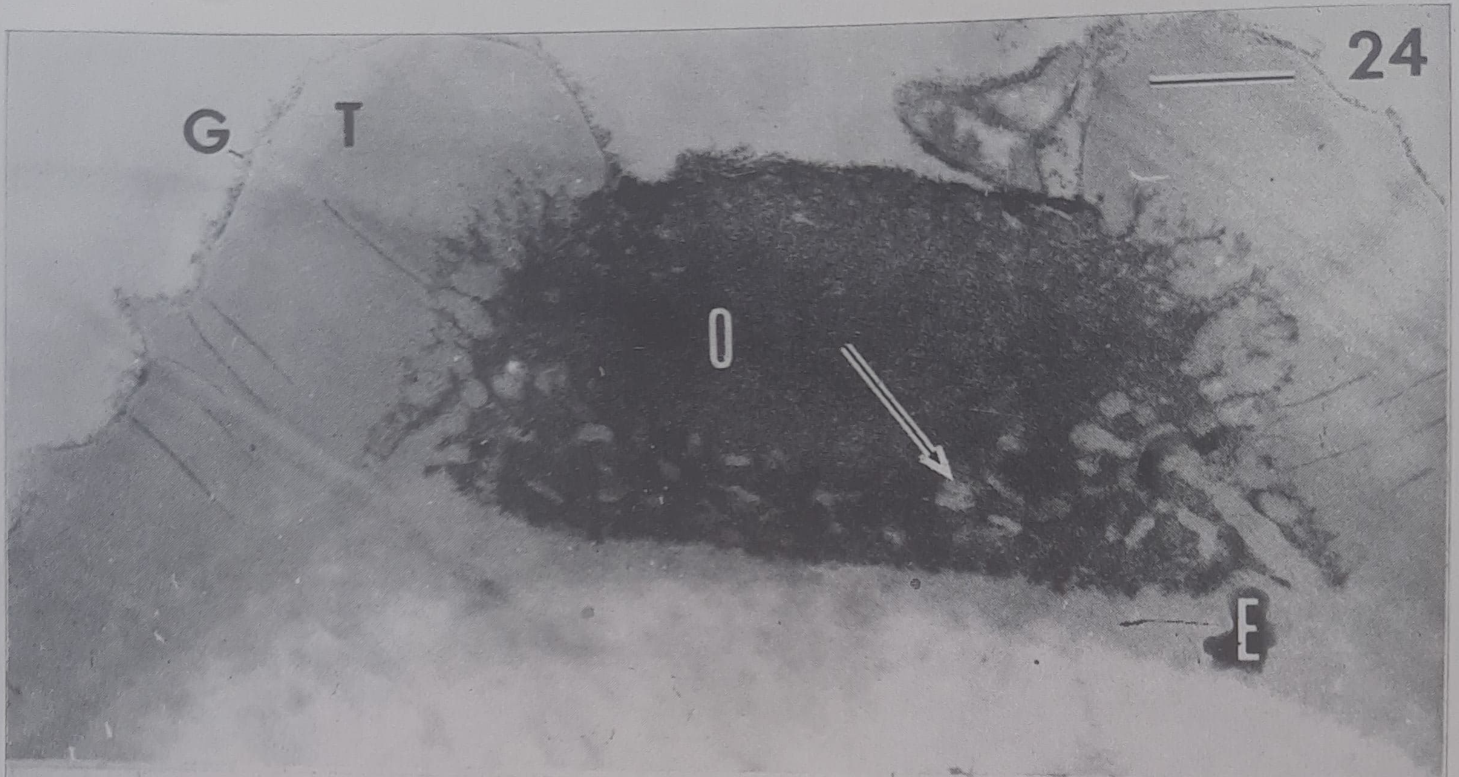

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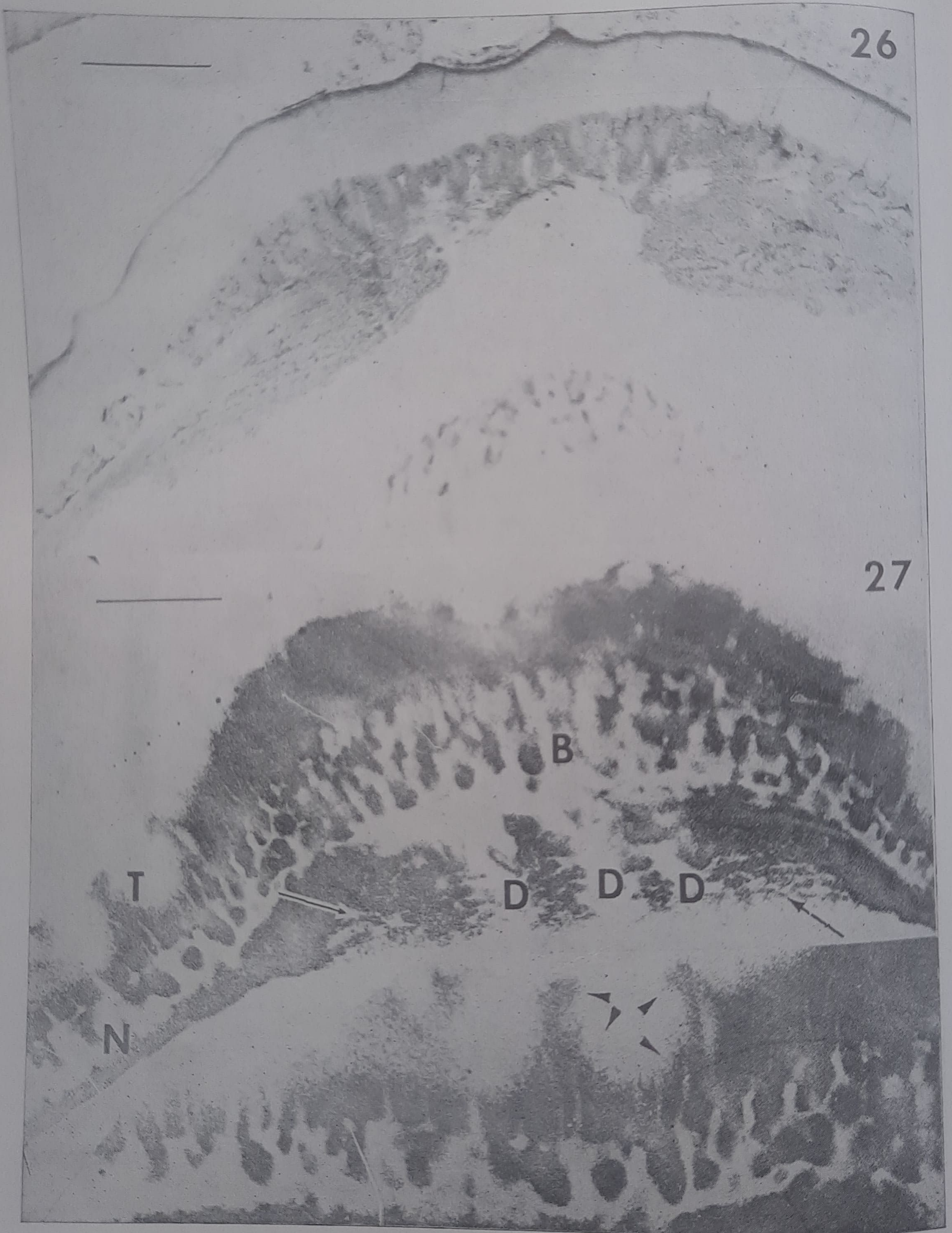
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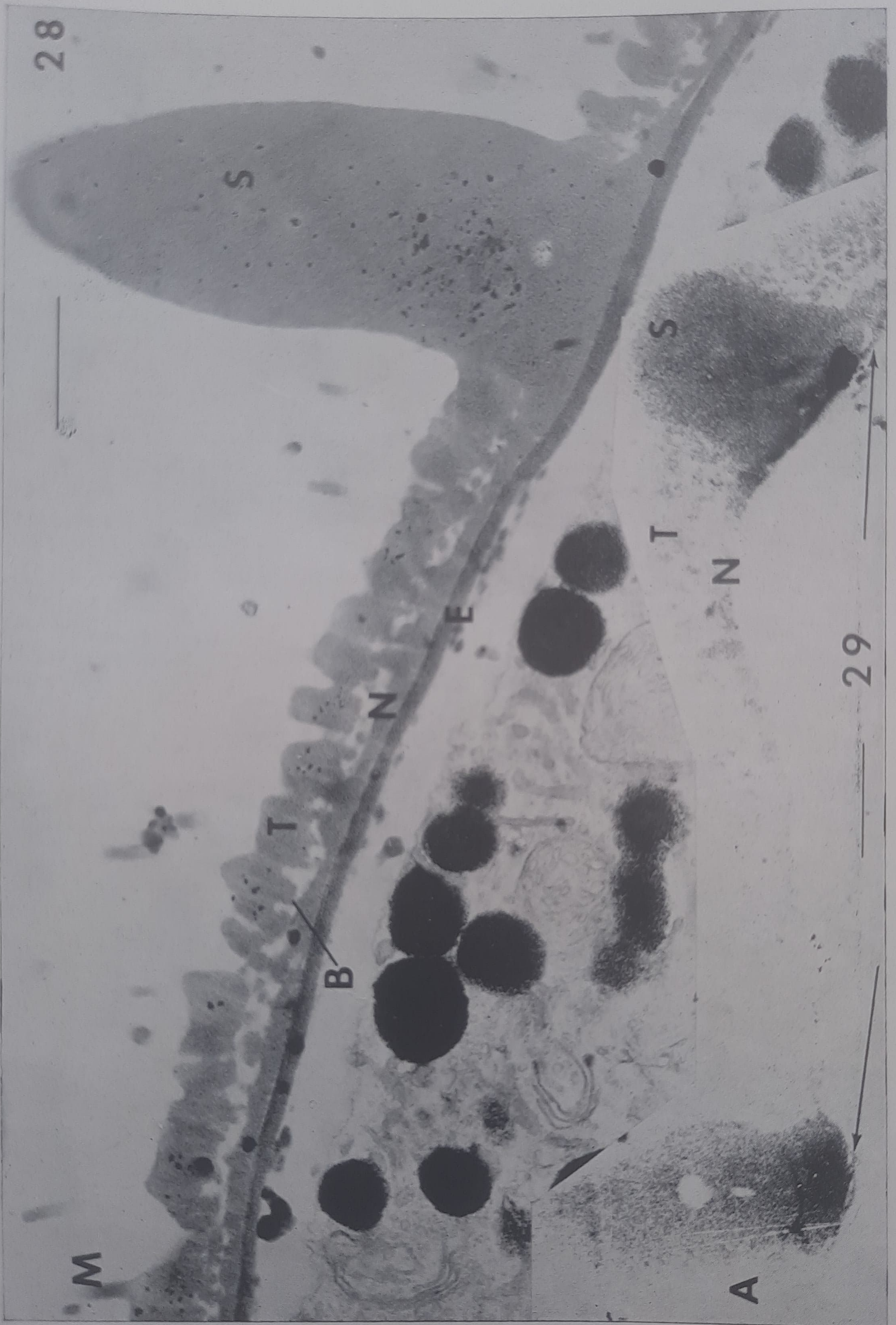
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23. An enlargement of the portion of exine located between the long arrows in Fig. 22. Part of the stain appears to be localized on numerous filaments (arrow heads) within the exine. Mag. scale: ca. 0.5 μm.

PLATE 13

24. Section of an aperture in pollen of *B. verrucosa* from an undehisced anther. Stained by PTA-chrom the microchannels across the tectum (T), the exine surface coating (G), and the oncus (O) are darkened. The exine is only weakly stained. The apertural lamellations of the endexine occur in the region marked "E" and within the oncus (arrow). The oncus recedes at pollen maturity and then endexinous lamellations appear as in Figs. 25-27. Treatment: none; Fixation: GA+barium carbonate; Stain: PTA-chrom. Mag. scale: ca. 0.5 μm.

25. In fresh mature pollen of *Betula* from dehisced anthers the exine is darkly stained by osmium-UA-Pb. Following treatment in KOH, the acetolysis mixture, or hot water staining by osmium-UA-Pb was greatly reduced and similar to the light portions of exines in Figs. 22 and 26. Treatment: none; Fixation: GA-OsO_4; Stain: UA-Pb. Mag. scale: ca. 0.5 μm.

PLATE 14

26. Staining was elevated in a marginal zone of the exine of fresh pollen of *Betula* following exposure to water. The differential stainability of the exine margin indicates either migration of exogenous substances, in this figure protein, into the exine or partial extraction of substances from the exine and their stabilization, perhaps as a result of chemical fixation, at the periphery of the exine. Treatment: hot water; Fixation: GA+barium carbonate; Stain: PTA-acetone. Mag. scale: ca. 1 μm.

27. The exine of *Betula* pollen grains was etched by successive treatment with acetol-KOH-Cl in any order. Destruction was greatest in the distal surface of the tectum (T) and nexine (N) in nonapertural regions of the exine. The filaments and white line centered lamellations (arrows) of the endexine in the apertural region are not destroyed. They may have been protected from concentrated reagents by the relatively small size of the aperture although extensive alteration of bacules (B) near by causes that explanation to be questionable. The disjunct nature of the endexine (D) is normal since lamellations of the endexine are like a fingered diaphragm in and under the oncus. In the enlarged insert of the etched tectum arrowheads indicate fine filaments exposed by destruction of exine matrix; weakly stained filaments cross the etched zones. Treatment: acetol-KOH-Cl; Fixation: GA; Stain: PTA-chrom. Mag. scale: ca. 1 μm.

PLATE 15

28. The exine of untreated pollen of *Nuphar luteum* has prominent spines (S), numerous spinules (M), a thick although incised tectum (T), short irregular bacules (B), and a nexine consisting of a foot layer (N) and endexine (E). Treatment: none; Fixation: GA-OsO_4; Stain: TCH-SP. Mag. scale: 1 μm.

29. Morphologically, selective destruction of the exine was similar after successive treatment in KOH-acetol-Cl, in any order, or in chromic acid. Except under spines the endexine was missing (arrows denote remnants of the endexine). Spinules were not apparent and the distal portion of spines was in many cases missing (S). The exine matrix was extensively removed from the tectum (T), bacules, and foot layer (N). The exine below the "A" is near the aperture margin. Treatment: chromic acid; Fixation: GA-OsO_4; Stain: UA-Pb. Mag. scale: 1 μm.