TRANSMISSION ELECTRON MICROSCOPY OF FOSSIL SPORES

by E. K. KEMPF

ABSTRACT. Transmission electron microscopy of fossil spores presents special difficulties. A major problem in megaspores is obtaining low power pictures of whole spores; this is overcome by using single-hole discs instead of grids. Care is needed to avoid damaging sporoderm ultrastructure by oxidation during preparation. Results achieved since 1965 are reviewed, and the sporoderm fine structures of *Setosisporites* (Carboniferous) as well as *Salvinia* and *Alnus* (both Tertiary) are illustrated and interpreted.

SEVEN years ago, in May 1965, the Department of Geology at the University of Cologne was provided with a transmission electron microscope, and a program was started to investigate the stratification and fine structure of fossil sporoderms in ultra-thin sections.

At that time there were only two papers published on this subject. Ehrlich and Hall (1959) studied some Eocene pollen grains without knowing, however, to what genera the material belonged. Pettitt and Chaloner (1964) tried to elucidate the fine structure of Mesozoic microspores from pollen sacs of *Cheirolepidium muensteri*, which are of the *Classopollis* type. Both publications demonstrated that it is possible and worth while to study fossil material in this manner.

TECHNICAL PROBLEMS AND SOLUTIONS

Our investigations were carried out step by step. The first task was to ascertain that the fine structure of sporoderms had been preserved in the fossil state without any change during fossilization. For this, comparison was necessary between the fine structural details of Recent and fossil sporoderms of the same species, and selection of suitable material turned out to be quite difficult.

Most of the papers published since 1952 on transmission electron microscopy of Recent sporoderms dealt with microspores, and especially with pollen grains. In preparing the material for such investigations, anthers from living or herbarium plants were reduced to small pieces, fixed, dehydrated, embedded in epoxy resins, and ultra-thin sectioned. There are, in comparison, only a few cases where fossil anthers, or parts of them, are found that may be treated in this way; for example *Classopollis* (Pettitt and Chaloner 1964) or *Alnus* (this paper). Most fossil microspores, however, occur dispersed and because of their extremely small size are not easy to handle. While the latter problem can be mastered using a micromanipulator, the main difficulty still remains: to determine the plant species in which the single microspore originated. In consequence of this uncertainty and as fine structural details are not very numerous in sporoderms of microspores, we decided to turn towards megaspores.

Cenozoic megaspores, which had been collected for biostratigraphical reasons, as well as Recent megaspores to serve as comparative material, were at hand. For the task in question Recent and Pleistocene megaspores of *Salvinia natans* seemed to be

[Palaeontology, Vol. 16, Part 4, 1973, pp. 787–797, pls. 101–103.]

most suitable. They were subjected to the usual sample preparation techniques and ultra-thin sectioned. The transmission electron micrographs gave a positive answer to our question. Sporoderm fine structure was preserved, and apart from some differences in electron density there was no change observable which could have been caused by fossilization. Other megaspores from Cenozoic, Mesozoic, and Palaeozoic strata were subsequently studied and corroborated the result.

The illustration of the findings for publication posed new difficulties. Because of the formvar film coated copper grids, which were used to support the ultra-thin sections, up to 50% of the large megaspore sections (about 0·5 mm in diameter) were hidden. In many of the ultra-thin sections it was possible to reveal the stratification and fine structure of a megaspore sporoderm, but the photographs are not suitable for publication. The paper of Pettitt (1966), which had been published in the meantime, also suffered from this difficulty. Indeed in most papers that dealt with transmission electron microscopy of sporoderms very tiny areas were figured at large magnifications, while figures at low magnifications giving a general view were missing or presented only as non-equivalent photo micrographs.

To give the maximum amount of information, best presentation and interpretation of sporoderm stratification and fine structure it seemed absolutely necessary to cover the whole field from very low up to the highest magnifications with transmission electron micrographs. Therefore we changed from copper grids to copper discs with one single hole of 400, 800, or even 1000 μm in diameter. To support the large ultra-thin sections it is necessary to reinforce the thin formvar films with which the holes were covered. It is then possible due to this method to get transmission electron micrographs at a magnification of about $\times 90$ with moderate resolving power, at magnifications of about $\times 450$ and $\times 1700$ with better resolving power, and above $\times 6000$ with high resolving power. Thus micrographs of single megaspore specimens in transmission electron microscopy can be produced, which nowadays, of course, should be supplemented by scanning electron micrographs.

Another difficulty is to find the best embedding medium for fossil spores. Because of the hardness of fossil sporopollenin and the necessity of good impregnation we use traditional methacrylate, in the formulation given below.

A further question is whether to stain the specimens or not. Until now we have avoided staining apart from some trials in order not to complicate our studies. In the future it will be necessary perhaps to use different stains, especially in microspores. Unstained specimens, should, however, always be studied for comparison.

We consider the demonstration that fossil sporoderms are often preserved in an excellent state as a main result of our studies. If however, one follows standard micropalaeontological or palynological techniques in which oxidizing chemicals are used, a certain amount of damage may occur. A most careful separation of the fossils from the embedding sediments is therefore required. In order to clean the specimens selected for study by the transmission electron microscope cold hydrofluoric acid (40%) is used, followed by washing with hot hydrochloric acid (25%) and distilled water.

Recapitulating, one can say that transmission electron microscopy of fossil spores at least requires the following treatment:

1. Careful separation from the embedding sediments, if possible without making use of oxidizing chemicals, in order to prevent damage.

2. Cleaning of the selected specimens by using cold hydrofluoric acid (40%), followed by washing with hot hydrochloric acid (25%) and distilled water.
3. Dehydration in a graded ethanol series followed by embedding in a 1:9 mixture of butyl/methyl methacrylate, containing 1% benzoyl peroxide. If different resins are used as embedding media, another kind of dehydration might be necessary.
4. After polymerization, cutting of ultra-thin sections on an ultra-microtome using glass and diamond knives. For magnifications up to $\times 10\,000$ mounting of ultra-thin sections should be done on formvar-film coated single-hole copper discs; for larger magnifications copper grids with or without film coating may result in higher resolving power.

STRATIFICATION AND FINE STRUCTURE OF FOSSIL SPORODERMS

Most of the results have been obtained from fossil megaspores, but some characteristic features of microspores are also presented.

For the interpretation of sporoderm stratification and fine structure it is necessary to make comparative studies of Recent spores in ultra-thin sections. Until now the resulting transmission electron micrographs never revealed more than three layers, which in my papers are named intine, exine, and perine, from inside to outside. It should be mentioned here that my terms exine and perine do not correspond with such terms of some other authors.

Cenozoic filicopsid megaspores and microspores. Most of the megaspores which have been found in Cenozoic strata belong to genera of the heterosporous ferns. Many species have been studied in ultra-thin sections, such as *Azolla* (Kempf 1969 *a*, *b*) and *Salvinia* (Kempf 1971*b*).

In fossil *Azolla* megaspores, two layers are preserved. The inner one, the exine, is quite thick, electron dense, and restricted to the rounded distal half of the megaspore. The outer layer, the perine, surrounds the exine. At the proximal pole it forms a large gula, to which the floats adhere via threads. The number of floats differs from subgenus to subgenus.

When, at larger magnifications, megaspore sporoderms of different species are compared with each other, the exines look quite similar. The fine structure of the perine, however, is heterogeneous and changes from species to species. It is therefore very useful for identifications on the species level. Within one species, the perine fine structure changes according to a zonation. In Miocene *Azolla nana* the structure resembles the differentiation into foot layer, bacula, and tectum of certain pollen grains. Furthermore there are fine structural differences between the distal and the proximal part, so that around the gula, the perine is quite different from that in the distal part of the megaspore. In *Azolla*, the intine is not preserved in the fossil state because it consists mainly of cellulosic material.

Hitherto, fossil intine has only been found in the megaspores of some species of *Salvinia*, e.g. *Salvinia natans*. The electron density of this material suggests that its preservation was made possible by a certain content of sporopollenin. The exine fine structure, as in *Azolla*, does not vary very much from species to species. The perine is again characterized by its zonation and great variability of fine structure.

Sometimes even the remains of the sporangioderm, which enveloped the megaspore, are preserved.

From *Salvinia rhenana* megaspores, we learned that the sporoderms can be damaged where oxidizing chemicals are used in the laboratory. Where H_2O_2 had been used to disintegrate the sample, the intine had disappeared and the exine revealed initial signs of corrosion. New samples, treated without oxidizing chemicals, released megaspores in which the exine and intine were very well preserved.

The Miocene *Salvinia cerebrata* was preserved as complete sori, which, after breaking open, yielded megaspores and microsporangia. A longitudinal section of a megaspore shows the dense exine and the general arrangement of the perine fine structure, which is most complicated at the proximal pole (Pl. 101, fig. 3). There the delicate gula is hidden by three large germinal valves. The scanning electron micrograph gives a three-dimensional impression of this region (Pl. 101, fig. 4). The perine fine structure resembles that of *Halletheca* from the Carboniferous (Taylor 1971).

Ultra-thin sections of complete microsporangia disclosed the characteristics of the microspores. In *Salvinia* all microspore exines of a microsporangium share a common perine, which in its fine structure resembles that of the corresponding megaspore. Within the perine mass the exines are arranged in large cavities near the surface, each of which is provided with a triradiate germination mark. The exines are very homogeneous, electron dense and therefore poor in fine structure. With the exception of a slight thickening towards the germinal suture, there is no sculpture on the exine surface.

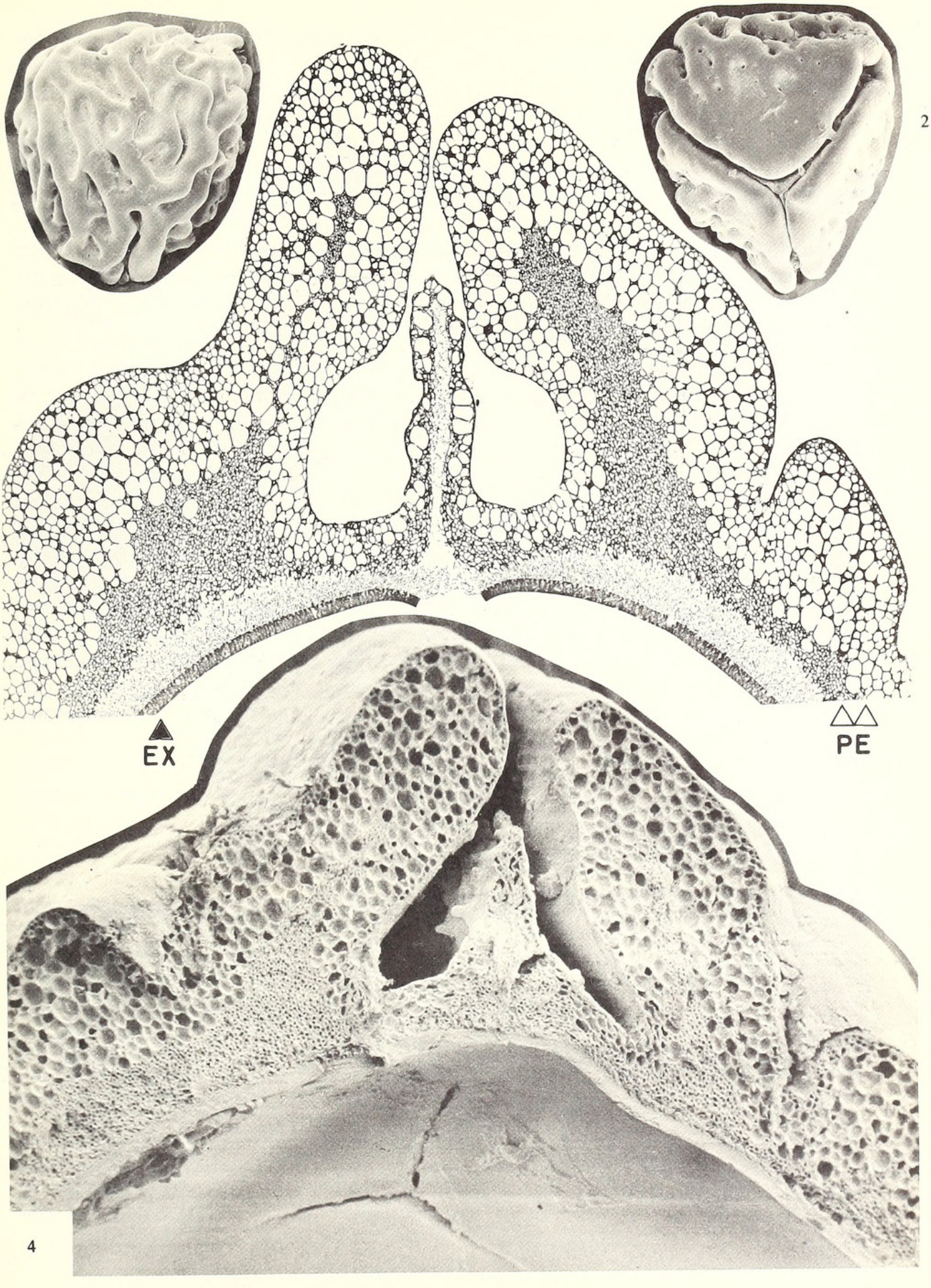
Cenozoic angiosperm pollen grains. As an example of fossil angiosperm pollen grains, pollen sacs of *Alnus* were embedded and ultra-thin sectioned. The material was collected from a clay lens within the lower-most part of the main seam of the Rhenish brown coal (Kempf 1971*b*), where it was found together with *Salvinia cerebrata*, amongst other plant fossils. The scanning electron micrograph of a piece of a pollen sac (Pl. 103, fig. 1) clearly demonstrates that these pollen grains represent the genus *Alnus*. As a dispersed spore, this type is known as *Alnipollenites metaplasmus* (Potonié 1931) Potonié 1960.

The transmission electron micrographs of ultra-thin sections (Pl. 103, figs. 2–4)

EXPLANATION OF PLATE 101

Megaspores of *Salvinia cerebrata*, Lower Miocene, W. Germany. Figs. 1, 2, 4: scanning electron micrographs; fig. 3: transmission electron micrograph.

1. Distal view; spore surface irregularly corrugated (cerebral sculpture), ×100.
2. Proximal view; triradiate gula with germination mark largely hidden by the three germinal valves, ×100.
3. Ultra-thin longitudinal section of proximal part showing two germinal valves and one ray of gula with germinal suture; exine (EX) relatively thin and quite dense; perine (PE) very thick and structurally subdivided in three zones (inner, middle, and outer zone); outer zone consisting of large, middle zone of small cavities; felt-like inner zone somewhat stretched because of laboratory treatment; ×675 (E 10009–10012, B 661, S 36393–2).
4. Proximal part of longitudinal half seen from inside; thin, dense exine showing triradiate germination mark on inner side; perine presenting two germinal valves and one ray of gula; spongy fine structure zonally differentiated, ×675.



KEMPF, Miocene megaspores

resemble those of Recent pollen grains of *Alnus* (Takeoka and Stix 1963). The sporoderm consists of two layers. The inner layer, the exine (= secondary exine, endexine, or nexine of other authors), is quite thin and electron dense. It is doubled in thickness over the arci, but looks like a porous membrane below the apertures. The perine (= primary exine, ektexine, or sexine of other authors) is formed by bacula and tectum. The bacula arise from the exine at irregular intervals, but are most numerous in the range of the arci. The coherent tectum is equatorially penetrated by the four large germinative pores and elsewhere by a great number of minute tubes (perpendicular bright lines). At each pore a vestibulum is formed by an arching upwards of the tectum. At the base of the bacula, exine and perine are fused together. There seems to be no foot layer; a condition recently described also for the Upper Cretaceous pollen grain *Wodehouseia spinata* (Leffingwell *et al.* 1970).

Mesozoic lycopsid megaspores and microspores. In Mesozoic nonmarine sediments, lycopsid megaspores are rather common microfossils. One of the first form species to be studied by us in ultra-thin sections was '*Horstisporites*' *semireticulatus* (Kempf 1971*a*). In this megaspore the sporoderm consists of a very heavy outer layer composed of ramifying sporopollenin threads—which was named perine, and a very thin laminated inner layer—which was named exine. Compared with the information at that time available about the sporoderm fine structure of Recent *Selaginella* megaspores (Martens 1960; Stainier 1965, 1967), this nomenclature seemed to be wrong.

It was necessary therefore to study some more Recent *Selaginella* megaspores (Kempf 1970), and this indicated that the naming of the layers in the fossil megaspore had been correct. In *Selaginella* the perine in fact is a very heavy layer with a variety of fine structure of different kinds, which mostly is arranged in concentric zones. The exine on the other hand is represented by a quite thin and laminated layer. The intine cannot be expected to be found in the fossil state since it is mainly composed of cellulosic substances.

Subsequently other Mesozoic megaspore species were examined in ultra-thin sections (Kempf 1972). A very thin laminated exine and a quite heavy perine, with

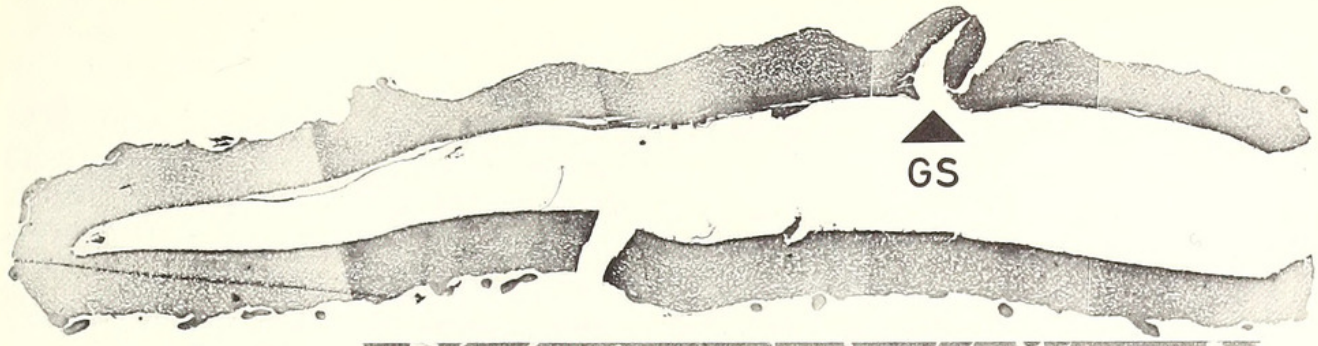
---

EXPLANATION OF PLATE 102

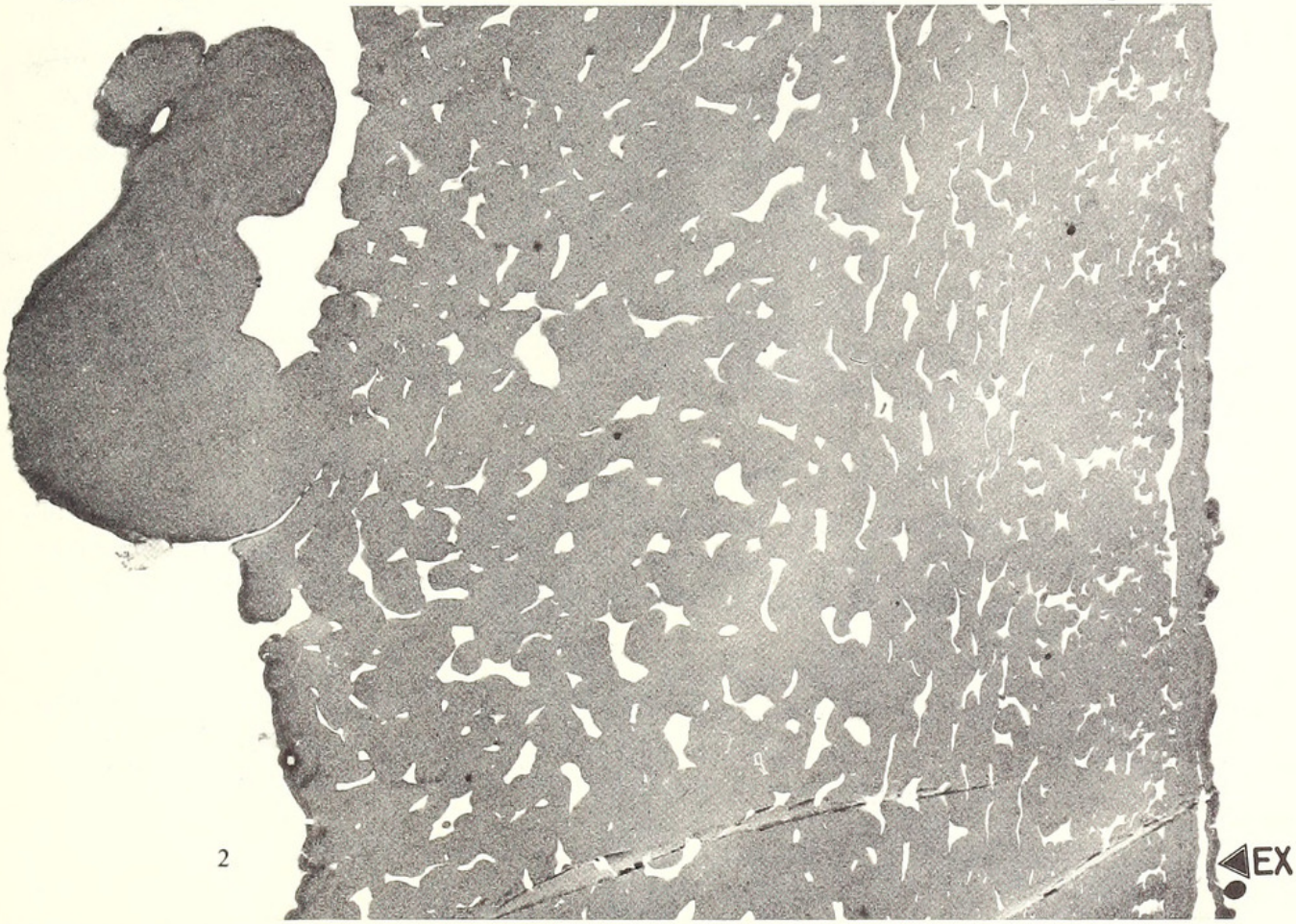
Figs. 1, 2. Megaspore *Setosisporites brevispinosus*, Namurian, Poland.

Fig. 3. Megaspore *Setosisporites hirsutus*, Westphalian B, W. Germany. All transmission electron micrographs.

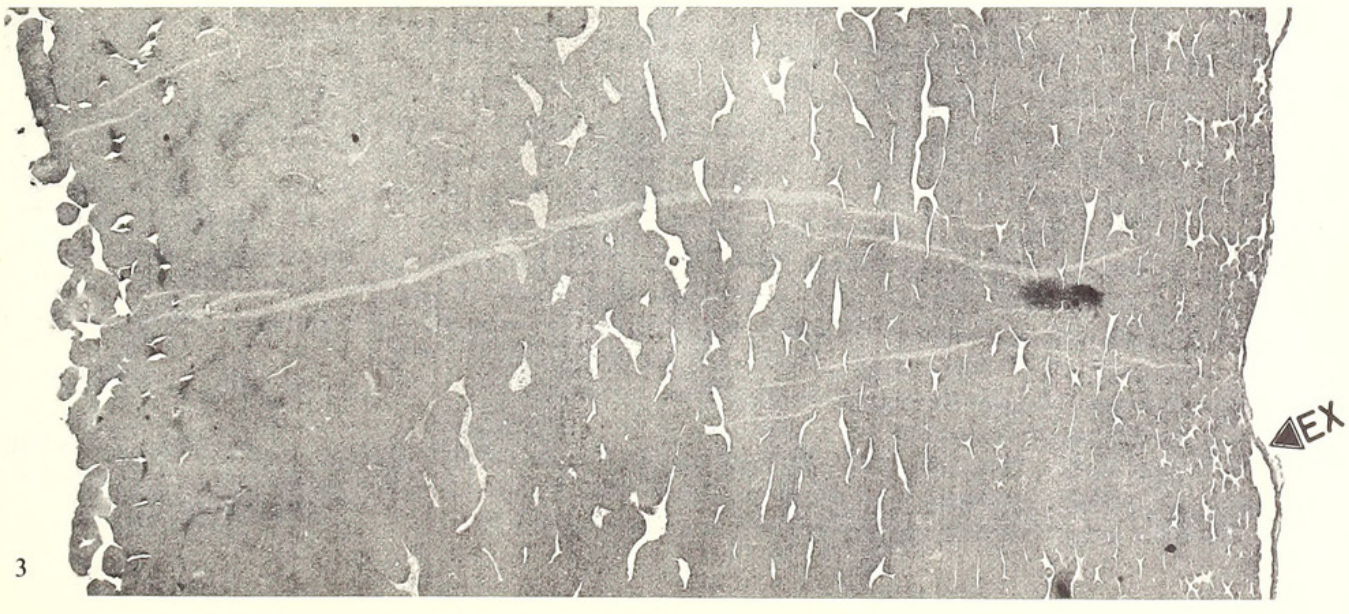
1. Ultra-thin longitudinal section of compressed specimen; proximal part with one ray of germinal suture (GS) and very thin exine which has loosened from inner side of perine; distal part with short spines rising from outer surface; inner third of perine more dense than outer two-thirds, indicating some kind of zonal differentiation in fine structure, ×340 (E 9329, B 740, S 36398–P 5).
2. Distal part of sporoderm in ultra-thin section; exine very thin and dense; it is doubled in this place as the exine cavity has collapsed; perine very thick with a fine structure formed by sporopollenin threads; a certain zonation in fine structure caused by variations in diameter or in main orientation; the spine regularly arises from the outer zone, ×6300 (E 9348, B 740, S 36398–Q 1).
3. Ultra-thin section of sporoderm; exine extremely thin; perine thicker by far with a zonal fine structure formed by sporopollenin threads; within inner and middle zone the threads are concentrically arranged but differing in diameter; within the large outer zone the threads form an irregular network the free space of which has partly been filled with an unknown substance during fossilization or laboratory treatment, ×5780 (E 8857, B 621, S 36397–G 4).



1



2



3

KEMPF, Carboniferous megaspores

fine structure formed by ramifying sporo-pollenin threads, were found in nearly all of these sporoderms.

Microspores were adhering to the surface of some of these megaspores and thus were sectioned by chance. They also consist of two layers—exine and perine—but it is not possible to detect any relationships to the most likely corresponding megaspore. Pettitt (1966) made the same observation when he compared the fine structures of Recent *Selaginella pulcherrima* megaspores and microspores.

In one type of megaspore a single thick layer only was encountered (Jux and Kempf 1971). Because the fine structure, which consists of radial tubes, differs from that of all other previously known megaspore sporoderms, a new form genus was created. This type of megaspore may perhaps represent some kind of Calamitacean plant.

Palaeozoic megaspores. The number of megaspore forms known from Palaeozoic deposits is very large. At the moment, however, it is quite difficult to find megaspores which are in such a good state of preservation that they are really suitable for ultra-thin section studies. We have attempted this with megaspores of *Setosisporites hirsutus* (Pl. 102, fig. 3) and *Setosisporites brevispinosus* (Pl. 102, figs. 1, 2). In fine structure they are similar to some of the Mesozoic megaspores, but it is obvious that the exine is extremely thin, while the perine shows an enormous thickness.

GENERAL TRENDS AND FUTURE WORK

It can be demonstrated that transmission electron microscopy of fossil spores provides us with a large amount of new and valuable information. Details, previously unknown or misinterpreted because of the limitations of optical microscopy, are revealed.

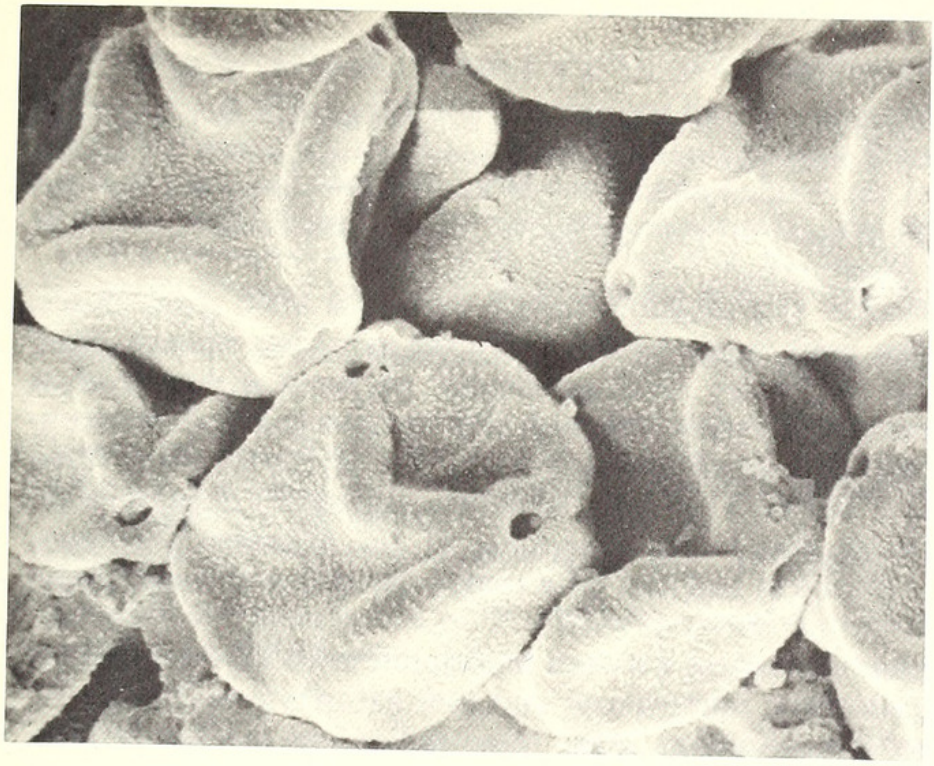
Of course most of the new information is relevant only to a single genus or species. There are, however, also general trends. One of these is the observation that in megaspores there is an obvious decrease in the ratio of perine to exine thickness, which is related to the state of evolutionary development of the megaspore (Kempf 1972).

EXPLANATION OF PLATE 103

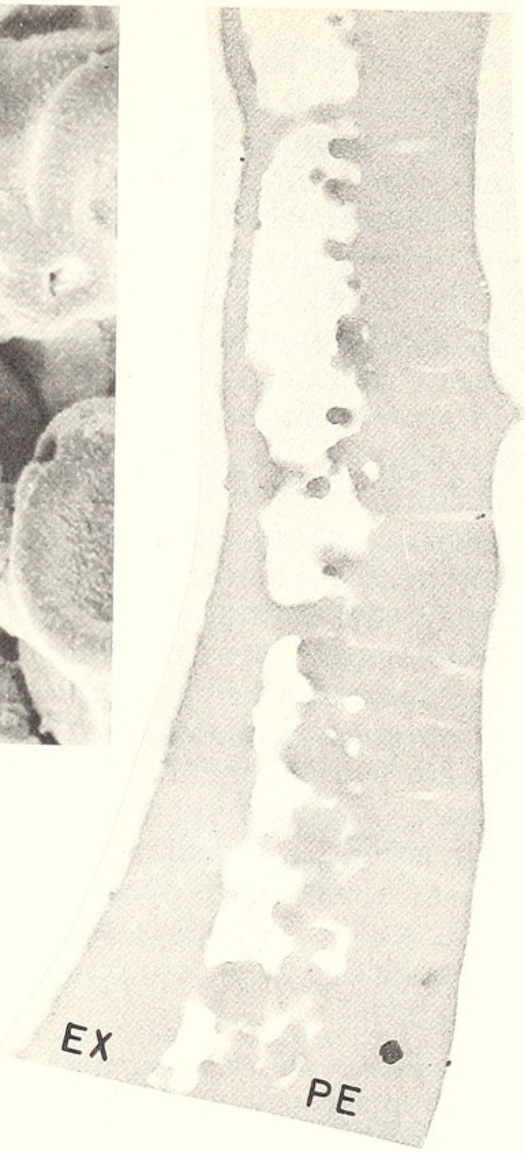
Pollen grains of *Alnus* ('*Alnipollenites metaplasmus*'), Lower Miocene, W. Germany.

Fig. 1. scanning electron micrograph; figs. 2–4: transmission electron micrographs.

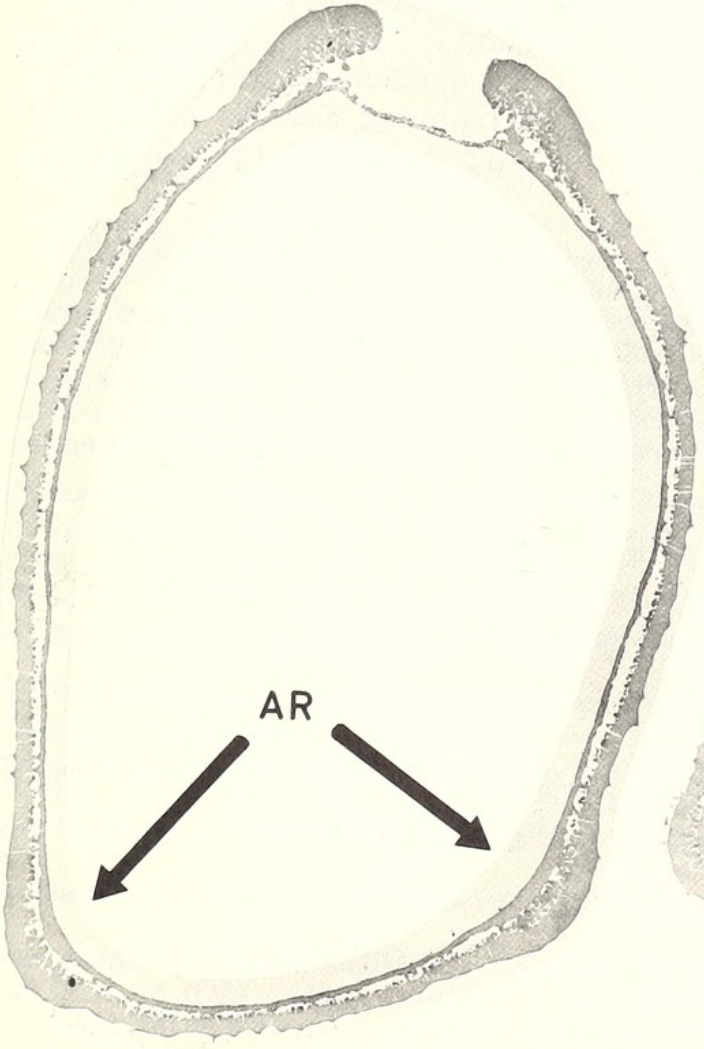
1. Part of pollen sac; all pollen grains with four equatorial apertures, which are connected with each other by two quite distinct arci; sporoderm surface provided with tiny spines, ×2000.
2. Ultra-thin section of pollen grain; sporoderm consists of exine and perine; exine very thin, dense and doubled in thickness in the area of the arci (AR), but like a porous membrane below the apertures; perine is formed by bacula and tectum; bacula rising from the exine at irregular intervals, most numerous in the range of the arci; the otherwise coherent tectum is penetrated only by the four large pores and by a great number of minute tubes (perpendicular bright lines); at each major pore a vestibulum is formed by arching upwards of the tectum, ×5500 (E 9899, B 771, S 62381–J 5).
3. Ultra-thin section of pollen grain, in which two pores and four arci were met with; ×3360 (E 9871, B 771, S 62381–J 2).
4. Ultra-thin section of pollen grain; sporoderm consists of exine and perine which at the base of the baculae are fused together; foot layer seems to be missing; perine is penetrated by minute tubes (perpendicular bright lines); ×28 000 (E 9897, B 771, S 62381–J 5).



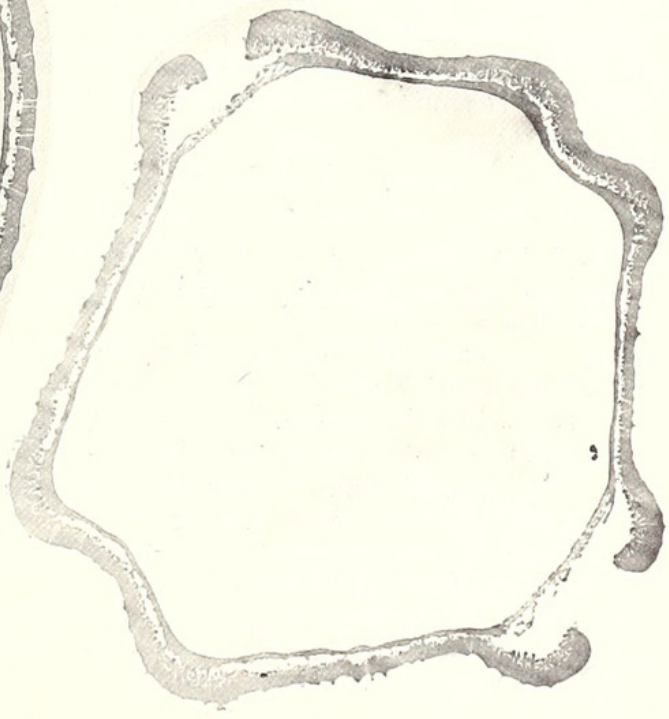
1



4



2



3

KEMPF, Miocene megaspores

One of the most important general results is a better knowledge of sporoderm stratification. It becomes apparent that all sporoderms comprise three layers: intine, exine, and perine. In the fossil state normally only two of them are preserved: exine and perine, with *Salvinia* as the single known exception. The fossilization of sporoderm layers depends on their content of sporopollenin. Where this is lacking the layer will also be missing in the fossil record.

The knowledge of sporoderm stratification, mainly obtained from megaspores, also can be applied to pollen grain sporoderms. There the exine has hitherto been named endexine, nexine, or secondary exine, while the perine was described as ektexine, sexine, or primary exine. In pollen grains it is sometimes not easy to recognize where the exine ends and the perine begins, as these two layers are often fused together as in *Alnus* (Pl. 103, fig. 4). There are, however, differences depending on function, which become most distinct in the range of the germinal openings. Apparently sporoderm stratification and fine structure are mainly subject to functional requirements.

Biologically, there are great differences between pteridophyte and spermatophyte megaspores, as well as pteridophyte microspores and spermatophyte pollen grains. However, if one considers the sporoderms alone these differences are less serious. Future work therefore should also consider the megaspore membranes of seed plants, as has been shown for Palaeozoic material by Zimmerman and Taylor (1971).

In general, it is demonstrable that in palynology and in other branches of palaeobotany a vast field is opened up by the use of the transmission electron microscope. Such studies nowadays should be completed by scanning electron microscopy, although due to its poor resolving power this method is incapable of providing sufficient detail.

Acknowledgements. The transmission electron microscope (Zeiss EM 9 A) and the instruments for preparative work (Leitz ultramicrotome, Leitz micromanipulator, etc.) were provided by the Deutsche Forschungsgemeinschaft. Mr. W. Mackowiak carried out the preparative work, as well as the analysis of ultra-thin sections in the electron microscope, with great skill. Megaspores of *Setosisporites hirsutus* and *Setosisporites brevispinosus* were presented by Dr. H. Grebe (Geological Survey, Krefeld) and Dr. A. Jachowicz (Geological Institute, Sosnowiec). The scanning electron micrographs were taken on a JEOL U 3 SEM (Kontron, LWU, Munich: operator Mrs. Liebel) by courtesy of Mr. G. Cichy (Kontron, Düsseldorf).

REFERENCES

EHRLICH, H. G. and HALL, J. W. 1959. The ultrastructure of Eocene pollen. *Grana palynologica*, **2**, 32–35, 2 pls.

JUX, U. and KEMPF, E. K. 1971. Microstructures of the Mesozoic megaspore *Tasmanitriletes* n. g. *Grana*, **11**, 95–100, 1 pl.

KEMPF, E. K. 1969*a*. Elektronenmikroskopie der Sporodermis von känozoischen Megasporen der Wasserfarn-Gattung *Azolla*. *Paläont. Z.* **43**, 95–108, 3 pls.

—— 1969*b*. Elektronenmikroskopie der Megasporen von *Azolla tegeliensis* aus dem Altpleistozän der Niederlande. *Palaeontographica*, **128 B**, 167–179, 8 pls.

—— 1970. Elektronenmikroskopie der Sporodermis von Megasporen der Gattung *Selaginella* (Pteridophyta). *Rev. Palaeobotan. Palynol.* **10**, 99–116, 3 pls.

—— 1971*a*. Electron microscopy of the megaspore *Horstisporites semireticulatus* from Liassic strata of Germany. *Grana*, **11**, 18–22, 1 pl.

—— 1971*b*. Elektronenmikroskopie der Sporodermis von Mega- und Mikrosporen der Pteridophyten-Gattung *Salvinia* aus dem Tertiär und Quartär Deutschlands. *Palaeontographica*, **136 B**, 47–70, 13 pls.

—— 1972. Electron microscopy of Mesozoic megaspores from Denmark. *Grana*, **11**, 151–163, 5 pls.

LEFFINGWELL, H. A. *et al.* 1970. A study of the fossil pollen *Wodehouseia spinata*. *Bull. Canad. Petrol. Geol.* **18,** 238–262, 7 pls.

MARTENS, P. 1960. Sur une structure microscopique orientée dans la paroi mégasporale d'une sélaginelle. Nouvelles observations sur la structure des parois mégasporales de *Selaginella myosurus* (Sow.) Alston. *Compt. Rend., Paris*, **250,** 1599–1602, 1774–1775, 2 pls.

PETTITT, J. M. 1966. Exine structure in some fossil and recent spores and pollen as revealed by light and electron microscopy. *Bull. Brit. Mus. (Nat. Hist.), Geol.* **13,** 221–257, 21 pls.

—— and CHALONER, W. G. 1964. The ultrastructure of the Mesozoic pollen *Classopollis*. *Pollen et Spores*, **6,** 611–620, 1 pl.

STAINIER, F. 1965. Structure et infrastructure des parois sporales chez deux sélaginelles (*Selaginella myosurus* et *S. kraussiana*). *Cellule*, **65,** 220–244, 5 pls.

—— 1967. Morphological study of the walls of the mega- and microspores of *Selaginella myosurus* (Sw.) Alston. *Rev. Palaeobot. Palynol.* **3,** 47–50, 1 pl.

TAKEOKA, M. and STIX, E. 1963. On the fine structure of the pollen walls in some Scandinavian Betulaceae. *Grana palynologica*, **4,** 161–188, 14 pls.

TAYLOR, T. N. 1971. *Halletheca reticulatus* gen. et sp. n.: a synangiate Pennsylvanian pteridosperm pollen organ. *Amer. Jl. Bot.* **58,** 300–308.

ZIMMERMAN, R. P. and TAYLOR, T. N. 1971. The ultrastructure of Paleozoic megaspores membranes. *Pollen et Spores*, **12,** 451–468, 6 pls.

E. K. KEMPF
Geologisch-Paläontologisches Institut
Universität Köln
D-5 Köln 1
Zulpicher Strasse 49
W. Germany

Revised typescript received 1 February 1973

Discussion on Dr. Kempf's paper:

Chaloner: In spores of fossil plants that you cannot immediately attribute to any group of living plants, e.g. to ferns or lycopods, how do you decide what is perine and what is exine?

Kempf: By analogy with spores of modern plants. There the exine plays no part in the ornamentation of the sporoderm surface. The ornamentation of the surface of a megaspore, for instance, is always made up by the perine. If the perine is composed of several zones, each zone follows the ornament observed at the surface. The exine, however, is unaffected. Further, the perine has a functional morphology; it has to protect the spore from the environment, and also it can fill its interstices with gas so that it can float on water.

Chaloner: It is a pity that we do not have 'types' in our terminology as well as in taxonomy. If we had a 'type' for the perine, it would surely be in the fern family Polypodiaceae. For most people, the term perine is more or less confined to this family. Other ferns, *Osmunda*, for example, do not have a perine. So that in *Osmunda* spores the exine must be forming the sculpture.

Kempf: I know of no instance where—according to transmission electron micrographs—the exine forms the sculpture.

Chaloner: How do you define exine then?

Kempf: It is the middle layer of a three-layered sporoderm.