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Texas Society for Electron Microscopy

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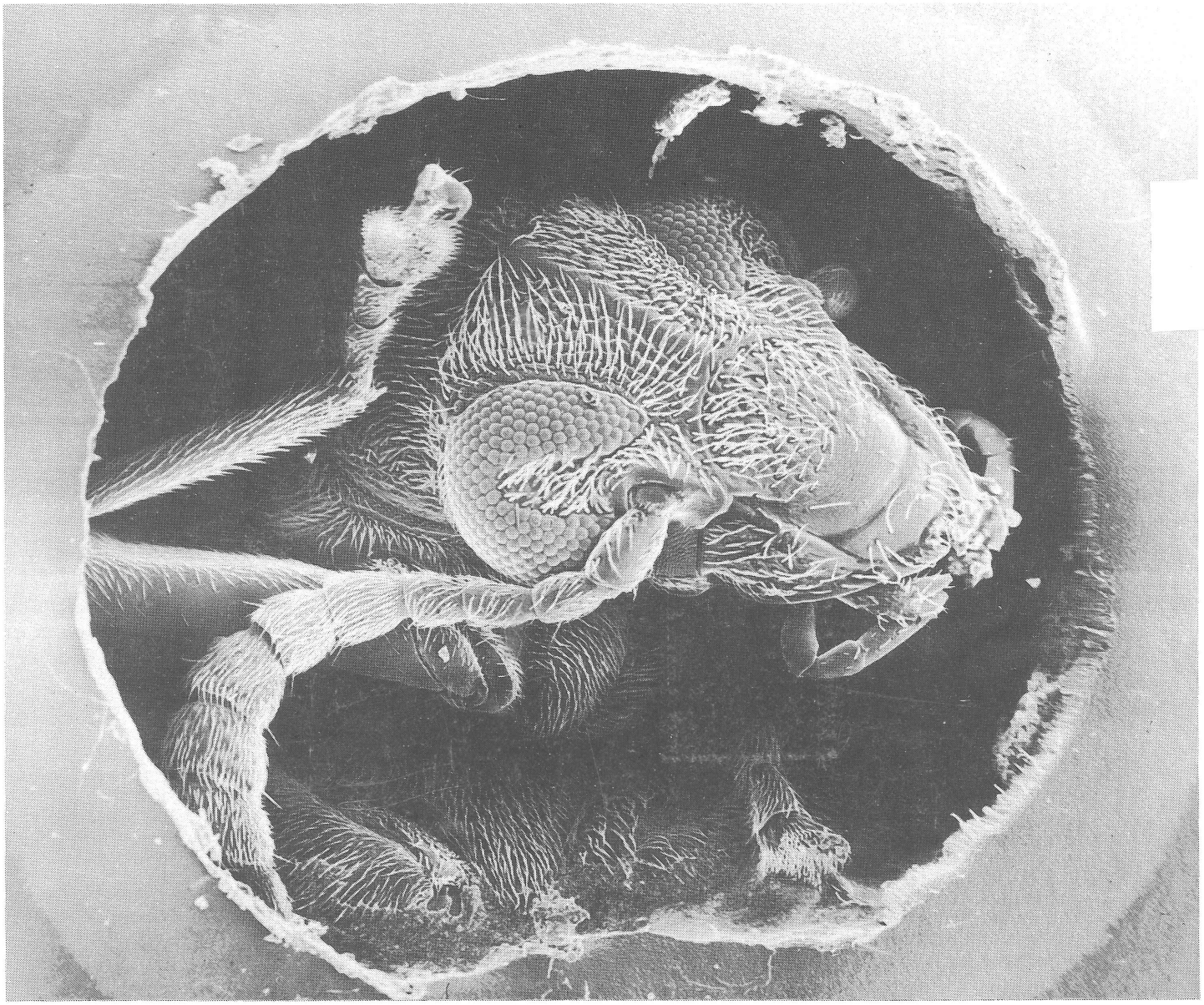

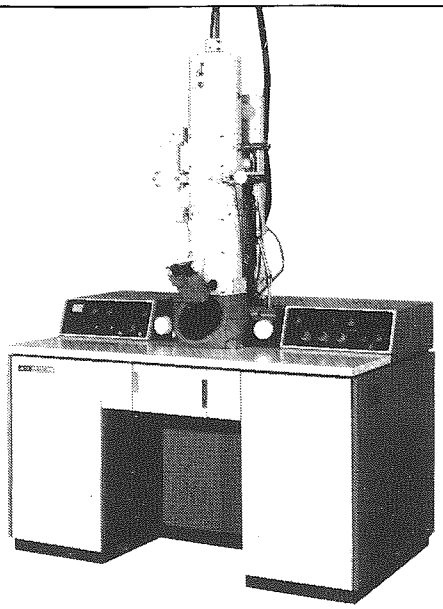

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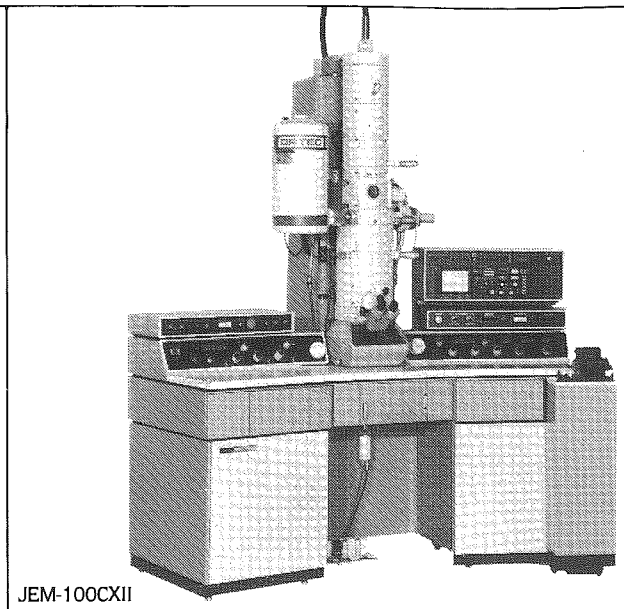

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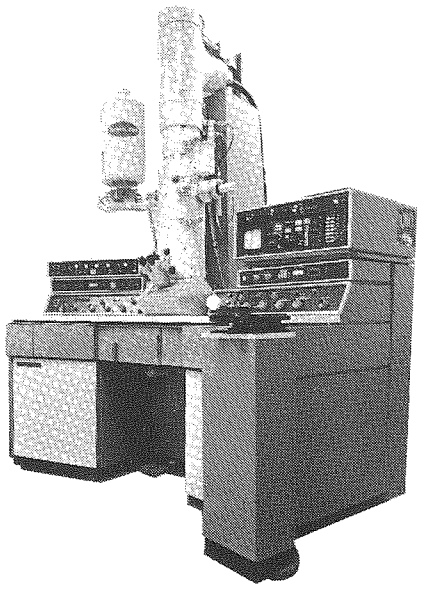

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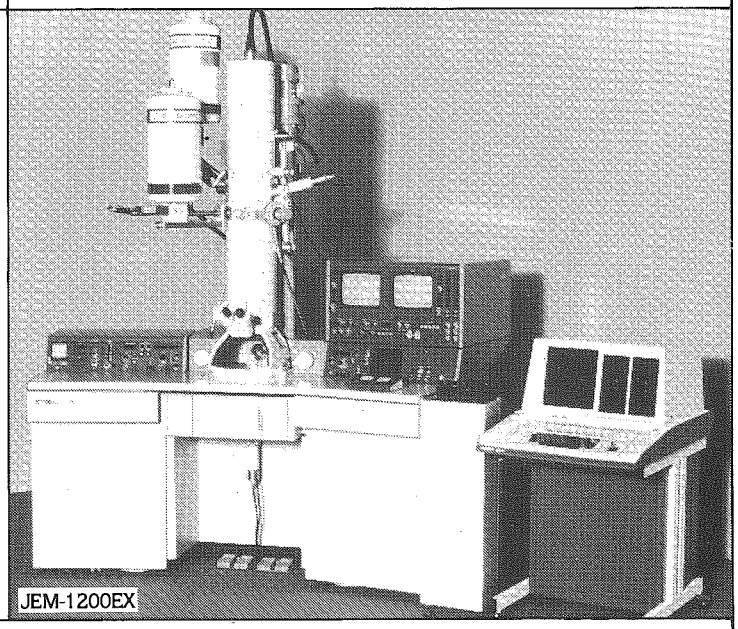

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Contents

JOURNAL
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Texas Society for Electron Microscopy

"For the purpose of dissemination of research with the electron microscope"

EDITORIAL POLICY

LETTERS TO THE EDITOR

Letters to the editor are printed as they are received in the order of their arrival. These letters reflect the opinion of the individual TSEM member and do not necessarily reflect the opinions of the editor or the society. The content of the letters should be concerned with the philosophical or operational aspects of the TSEM, the Journal and its contents, academic or national policies as they apply to TSEM and/or its members and electron microscopy in general. Editorial privilege may be evoked to insure that the LETTERS SECTION will neither be used as a political forum nor violate the memberships' trust.

ELECTRON MICROGRAPHS AND COVER PHOTOS

Micrographs submitted for Cover Photos should be marked as such. The choice of photographs will be made by the editor. Photograph receipt and/or dispensation will not be acknowledged. Photographs will not be returned. Electron micrographs to be used for cover photos and text fillers (interesting micrographs) are welcome and should be selected with some attention to aesthetic appeal as well as excellence both in technique and in scientific information content.

(Continued On Page 25)

ON THE COVER

Gibbobruchus mimus, a bruchid beetle which attacks seeds of **Cercis canadensis**. The developing beetle has fed on the inner tissues of the seed and, now mature, was preparing to exit through the hole it has cut in the seed coat using its specially adapted mandibles. The protective sclereid layers of the seed coat are visible at one side of the hole. Insects which attack seeds must be able in some way to penetrate the seed coat. By: M.A. Webb and H.J. Arnott — Biology Department — U.T.,Arlington.

President's Message

This is the final letter I will be writing to TSEM members during my term of office, so it is an appropriate time to thank you all for what has been a stimulating highlight to my years as a TSEM member. As I said when I took office, I am convinced that our society is not just the biggest but also the best of the local EM societies. The quality of the material presented at our meetings is outstanding, and the TSEM Journal continues to set the standard. Negotiations are currently underway to increase the distribution of the Journal and include the abstracts of meetings of some of the other local societies, and I urge each of you to give thought to contributing to the Journal by submitting papers, technical notes, etc.

Discussions are also being held with our colleagues in Louisiana in the hope that we can arrange joint meetings between our two societies, similar to those held in the past. As many of you know, the main reason it was found necessary to discontinue these meetings was the expense incurred by a TSEM member participating in a meeting in the French Quarter of New Orleans, or by LSEM members attending one held in an expensive hotel in a central Texas city. If we can select sites close to the state line, and provided the local arrangements committee is cost conscious, it should be possible to resume our joint meetings. If you have attended them in the past, you will recall how informative and enjoyable they invariably were.

I am pleased to inform those of you who have not already heard that the Society has conferred Honorary Membership on Larry Thurston. We continue to hear encouraging news of his slow but steady progress.

My term as president has been a relatively painless one, and this is largely due to the excellent support I have had from other members of the executive council. It is a pleasure to thank them all.

Bruce Mackay
President TSEM
1982-1983

President Elect's Message

At last year's Spring meeting of TSEM an **ad hoc** committee was appointed to study the status of our corporate members. A questionaire was devised and mailed to each corporate member in an attempt to obtain feedback. Topics considered included such things as meeting locations and formats, advertisements in the TSEM Journal and general policies regarding corporate members. Overall the responses were quite positive although there are a few areas that appear to be in need of attention. I thought it would be appropriate for the committee to share some of the information gathered with the general membership by means of this letter.

Most of the corporate members who responded to our questionnaire appear to be pleased with TSEM. Dues seem to be reasonable and most are pleased with the Journal and the policies pertaining to advertisement therein. Corporate members are evenly divided on whether we should meet in larger cities or continue to choose smaller cities as we have been doing recently. All want to see larger "turnouts' for the meetings and most favored more joint meetings with other EM groups.

In reading the various responses, I should note that some of our corporate members appear to feel that the general membership does not take full advantage of the presence of corporate representatives at our meetings. Perhaps the following statement from one of the questionnaires will help to convey this feeling better that I can. "I would like to see more participation of the attending members in the corporate exhibit area. We don't bite. You might be surprised that some of us can make good, intelligent conversation, and provide sound scientific information on events and techniques in our own areas of expertise. I don't mean to sound so cynical but often when I stand for a whole day in my booth at a TSEM meeting, I see very few people come by and just talk."

In closing I should emphasize that the Council is always open to suggestions from all TSEM members on all aspects of our business. In this case we are particularly interested in any suggestion you may have as to how we can make TSEM more attractive to corporate members. Corporate members are most important to the future of TSEM and we benefit from their participation in numerous ways.

Sincerely,

Charles W. Mims
President Elect, TSEM

Editor's Message

This letter will delineate TSEMJ's planned yearly issue schedule. Though we've more or less held this order for several years, now seems the time to finally put it in press. The schedule should prove helpful to readers and contributors alike!

Issues two and four of each volume will embody our meeting abstracts and a minimum of two contributed articles (reports, reviews, or technical notes). Issue one will present three or more proffered articles and the Society's By laws. The membership list will appear in the fourth issue each year. The third issue is, as of now, tentatively targeted at publishing abstracts from other regional EM meetings held throughout the country. The new participants (advertisers and societies) will bear the biggest burden of added printing costs.

TSEM will benefit at least three ways from this endeavor. As a forum for other regional meeting abstracts, TSEMJ will be able to disseminate new and diverse research information to its various readers. Such abstract data usually preceeds manuscript publication by several months. TSEMJ's circulation will greatly expand and we'll enjoy national and even international exposure from this enterprise. As mentioned, additional financial obligations of this issue should almost be alleviated from the TSEM. The whole concept sounds exciting to me.

I remain in need of manuscripts from prospective contributers. It should be obvious by now that the editor (T.E.) has to wait for the arrival of such text before he or she can assemble them into a viable Journal issue. When (note the "when") you do sent in a manuscript, be sure to give the editor plenty of lead time. The papers have to be subjected to the review process and that usually takes several weeks. Don't be shy with your contributions, T.E. will help you get them into press.

A reminder that selection to the TSEMJ review committee is by overall society participation (papers, abstracts, and/or personal contributions). If you are not active, you won't stay on the committee; if your activity stirs, you'll find yourself with the committee assignment.

In conclusion I'd like to make two statements; "Last minute journalism is for the birds" and "Don't complain about TSEM or TSEMJ unless you've carried your share of the LOAD." "If you have, then go for it!!!"

My best wishes to you all.

Paul S. Baur
Editor, TSEMJ

TSEM Minutes

The TSEM Business meeting was called to order by President Mackay at 8:20 p.m., October 8, 1982 in the Hotel Galvez, Galveston, Texas.

1. The minutes were read and approved.
2. The treasurer's report was given and accepted.
3. A report on the membership was made by the secretary.
4. News concerning the condition of Larry Thurston was given by J. Randy Scott. Larry still has severe motor impairment resulting from the accident, but now has use of his left arm and is still making progress. He remembers everyone and enjoys receiving cards and letters from TSEM members.
5. Results from a questionnaire sent to corporate members were reported by Charles Mims. There was a 50% response to the questionnaire, and the majority indicated satisfaction with most aspects of their relationship to TSEM. Thanks were extending to corporate members for their participation in the society.
6. A report on the past EMSA meeting was made by Bruce Mackay.
7. Dr. Lee Peachey, president of EMSA and speaker at the meeting, characterized the relationship of EMSA to its local affiliates, of which TSEM is the largest. He invited TSEM members to join EMSA and become active in the national society.
8. A report on the TSEM Journal was made by Paul Baur, editor. More advertising is needed for the next issue of the Journal. Members are urged to support the advertisers. Members are invited to submit papers to TSEM Journal. All papers are reviewed. Persons who are not TSEM members may join as they submit their papers.
9. Information about the spring meeting was given by Pat Davis. The meeting will be held in Austin, Texas on April 7, 8 and 9 at the Quality Inn on South Interregional Highway, Interstate 35. Activities on the campus of The University of Texas at Austin are planned for Thursday afternoon.
10. Dr. Mackay thanked Pat Davis and the local arrangements committee.

The meeting was adjourned at 8:40 p.m.

Respectfully submitted,
Elizabeth J. Root, Ph.D.
Secretary, TSEM

Regional Editors

Lynn Blum, Department of Neurobiology and Anatomy, University of Texas Medical School at Houston, P.O. Box 20708, Houston, TX 77025. (713) 792-5700.

James K. Butler, Department of Biology, The University of Texas at Arlington, Arlington, TX 76010. (817) 273-2871.

Bernell Dalley, Department of Anatomy. Texas Tech University School of Medicine, Lubbock, TX 79409. (806) 742.5277.

Lynn Davis, Department of Veterinary Anatomy, College of Veterinary Anatomy, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843.

Joanne T. Ellzey, Biological Sciences, The University of Texas at El Paso, El Paso, TX 79968. (915) 749-5609.

Cheryl M. Craft, The University of Texas, Health Science Center at San Antonio. 7703 Floyd Curl Drive, San Antonio, Texas 78284.

Randy Moore, Department of Biology, Baylor University, Waco, TX 76798. (817) 755-2911.

Elizabeth Root, GEA 115, University of Texas, Austin, TX 78712.

Anna Siler, Department of Pathology, The University of Texas Southwestern Medical School, Dallas, TX 75235. (214) 631-3220.

David L. Murphy, Department of Medicine, Baylor College of Medicine, Texas Medical Center, Houston, TX 77030. (713) 790-3146.

Marilyn Smith, Department of Biology, Texas Women's University, Denton, TX 76204. (817) 566-6400.

Glenn Williams, The University of Texas Health Center at Tyler, P.O. Box 2003, Tyler, TX 75710. (214) 877-3451.

Financial Report

ASSETS ON MARCH 15, 1983		
Certificate of Deposit No. 91099, Univ. Natl. Bank, Galveston	$ 2,000.00	
Certificate of Deposit No. 10-141345, Houston First Savings	2,456.02	
Merrill Lynch Money Market, Dallas	2,000.00	
Checking Account, Forestwood Natl. Bank, Dallas	4,180.94	
Includes Paul Enos Memorial Fund - $50.00	10,636.96	$10,636.96
RECEIPTS		
Denton Meeting - Registration	1,962.00	
Interest		
CD No. 92099	87.50	
CD No. 10-141345	124.68	
Merrill Lynch	168.54	
Checking Account	170.33	
Secretarial (Smith) Close Account	9.34	
Memberships	1,016.00	
	3,538.39	3,538.39 +
DISBURSEMENTS:		
Denton Meeting		
Social	777.64	
Banquet	743.50	
Council Luncheon	60.50	
Coffee/Donuts	79.63	
Transportation	99.17	
Guest Speaker	225.65	
Entertain Guest Speaker	102.65	
Mixer	78.53	
Presidential (Baur) Honorarium	100.00	
Miscellaneous	11.41	
Student Travel	131.00	
Plaques	213.30	
Secretarial (Smith) Expenses	135.00	
Income Tax Preparation	160.00	
Secretarial (Root) Expenses	680.00	
"Larry Thurston Computer Fund"	200.00	
Treasurer Expenses	6.13	
TSEM Journal Printing	800.00	
Presidential (Mackay) Travel-EMSA Washington	269.40	
	4,873.76	4,873.76 –
ASSETS ON OCTOBER 4, 1982		
Certificate of Deposit No. 91099, Univ. Natl. Bank, Galveston	2,000.00	
Certificate of Deposit No. 10-141345, Houston First Savings	2,580.70	
Checking Account, Forestwood Natl. Bank, Dallas	4,720.89	
Includes Paul Enos Memorial Fund ($50.00) and Merrill Lynch Money Market Cashed ($2,016.36)	9,301.59	9,301.59

Respectfully submitted: W. Allen Shannon, Jr., Treasurer

EXPERIMENTAL INOCULATION OF HELA CELLS WITH GRAM-NEGATIVE BACTERIA: AN ELECTRON MICROSCOPICAL STUDY

by

Gabriel Seman, M.D. and Carolyn E. Cook, B.A.

Department of Tumor Virology
The University of Texas
M.D. Anderson Hospital and Tumor Institute at Houston
Houston, Texas 77030 U.S.A.

Request reprints from:

Gabriel Seman, M.D.
Associate Virologist
The Department of Tumor Virology
The University of Texas System Cancer Center
M.D. Anderson Hospital and Tumor Institute
6723 Bertner Avenue
Houston, Texas 77030
(713) 792-3630

INTRODUCTION

In a previous paper (1), we have described an unusual case of bacterial contamination in an antibiotic-free tissue culture of pleural effusion cells from a patient with breast cancer. The case was unusual in that bacteria grew in the cytoplasm of the cells, accumulating into often huge cytoplasmic blebs released into the medium, thus perpetuating the infectious cycle. Infected cells grew vigorously for many weeks, but with time became more and more sluggish and stopped growing in passage 7, about seven months after initiation of the cultures. The other unusual aspect of this contamination was that it proved impossible to isolate the bacteria (which were gram-negative) on any of a number of bacteriological media. Their replication appeared entirely cell dependent. In this paper, we report the successful infection of HeLa Cells by these bacteria. The morphological patterns of this infection were the same as those seen in pleural effusion cells. HeLa cells entrapped the bacteria by simple phagocytosis. The addition of penicillin and streptomycin to infected HeLa cultures produced severe bacterial damage but did not eradicate the microorganisms, which still remain uncharacterized.

MATERIALS AND METHODS

Uncloned HeLa cell cultures maintained for several years in our laboratory were used.

To infect HeLa cells, cells in T-25 and T-75 Corning plastic flasks were inoculated with 2 ml of medium from infected pleural effusion cell cultures. The medium was gently centrifuged (500 rpm for 5 min. to remove cells but not bacteria, and the supernatant used for inoculations. Infected HeLa cultures were grown without antibiotics in Leibovitz's L15 medium and incubated in a dry, air flushed incubator at 37.5 C.

To maintain the infected cultures, the medium of the original flasks was renewed every week after gently shaking the flasks and discarding the floating elements; infected cells grew back rapidly every time. The flasks were kept in this way for four months, during which time parts of the floating elements were also used to seed new flasks. Infected cells were thus passaged four times in four months. No flasks were lost to acute infection, and culture medium remained clear throughout. Cell growth and infection were maintained in a steady state by both methods.

To study the mechanism of infection, fresh HeLa cells were plated into a T-75 flask together with 2 ml of infected medium from a pleural effusion cell culture in passage 6. Half of the monolayer, which grew rapidly, was scraped 7 days after inoculation for examination by electron microscopy. Cells and medium were pelleted at 1000 rpm for 10 min. and after fixation in glutaraldehyde and osmium tetroxide embedded in epon-araldite.

To study the effect of penicillin and streptomycin, 30 ml of medium containing 500 units of penicillin and 100 micrograms of streptomycin per ml were added to a T-75 flask in which infected HeLa cells were growing for 20 days. Antibiotic-containing medium was changed twice, during a period of 20 additional

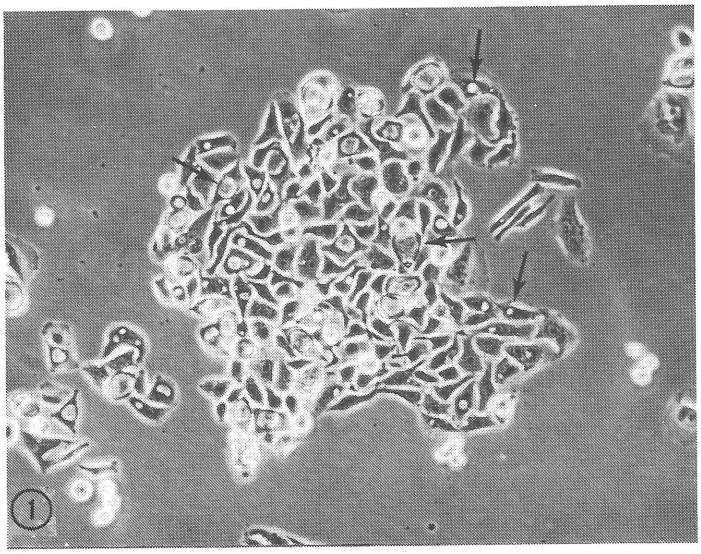


FIGURE 1: Colony of HeLa cells 7 days after inoculation with bacteria. Many cells contain vacuoles (arrows). Phase contrast, 140x.

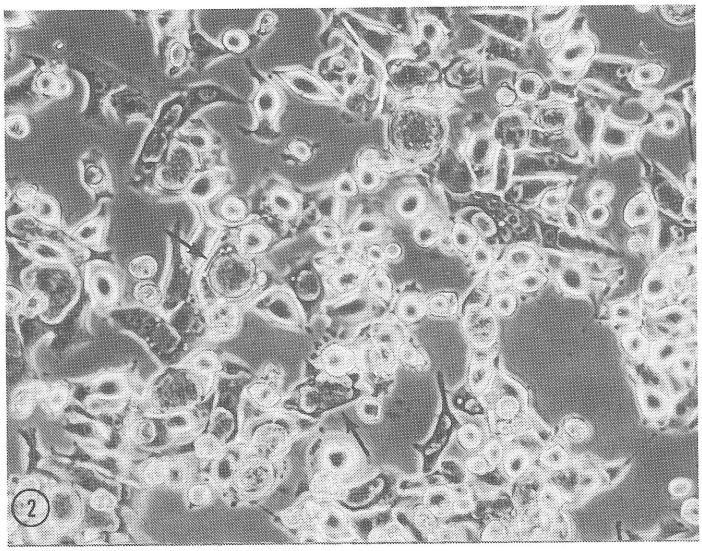


FIGURE 2: HeLa cells 7 days after infection. Most intracellular bacteria-laden vacuoles are large in size (arrows). Phase contrast, 160x.

days, by shaking the flask and discarding the floating elements, after which the treated culture was examined by electron microscoy.

RESULTS

A few days after plating, infected HeLa cells formed colonies in which many cells contained small, rounded vacuoles (Fig. 1). With time, most of the cells showed one or several, often very large, vacuoles (Fig. 2).

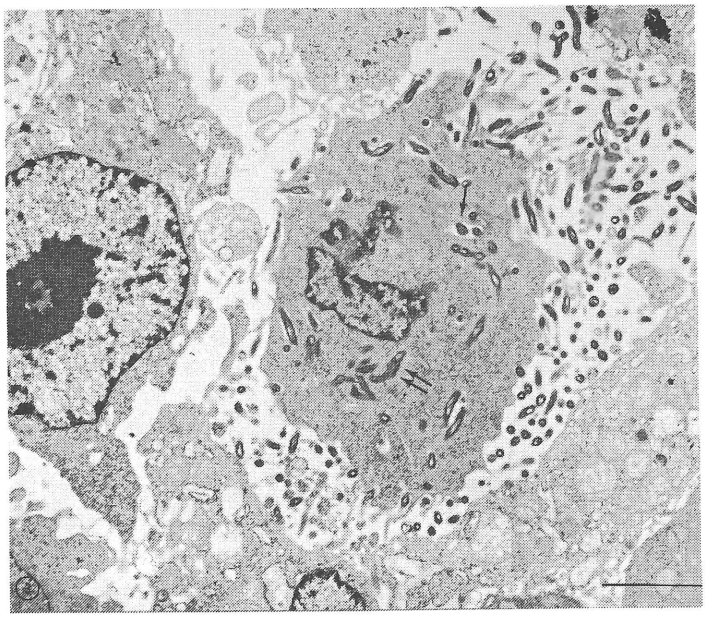
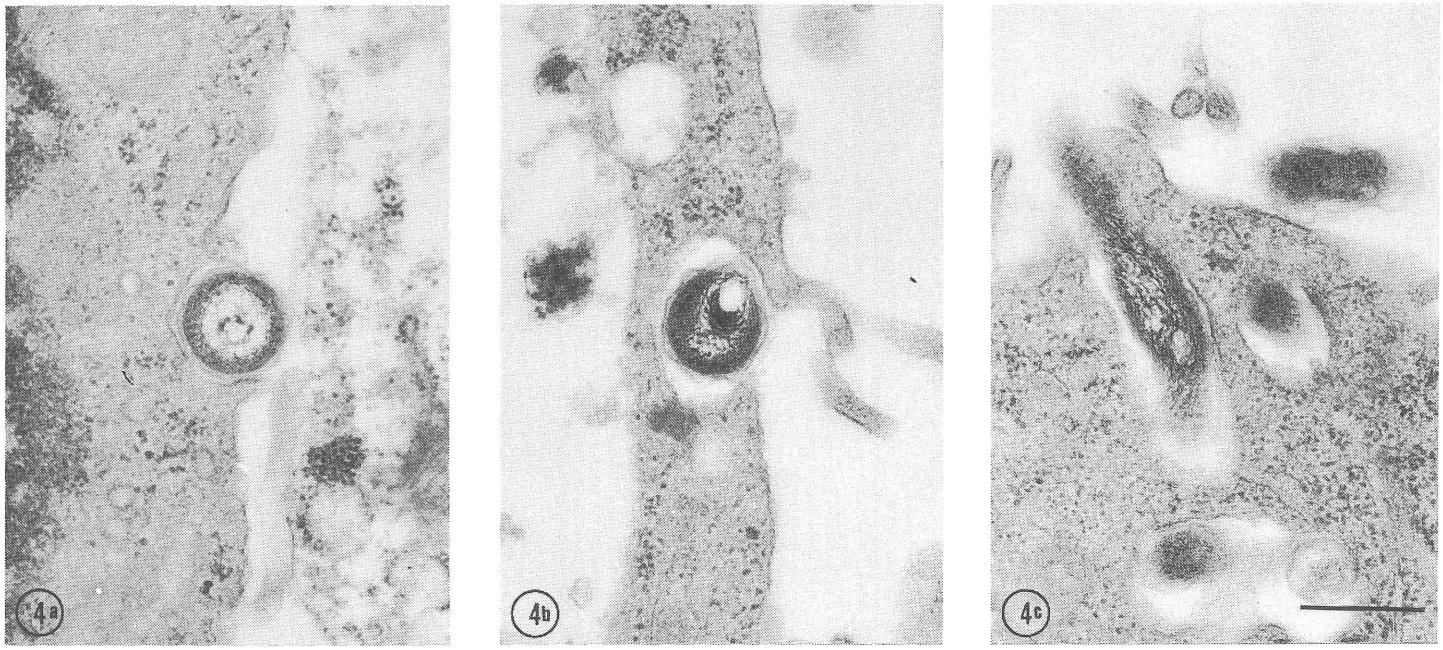


FIGURE 3: A young HeLa cell besieged by bacteria. The microorganisms are picked up mostly one by one at the cell periphery, ending up in vacuoles (arrow) or in plain cytoplasm (double arrows). Line: 4μm.

In these experiments, we were essentially concerned in knowing how HeLa cells became infected. A HeLa cell culture examined in the electron microscope 7 days after infection revealed that the mechanism of infection was that of simple phagocytosis. Extracellular bacteria were trapped in the cytoplasm of the cells after being engulfed by pseudopod-like processes (Figures 3 and 4a, b, c). Engulfment seemingly did not involve special adhesion sites between cells and bacteria, or modifications of the cell membrane facing the bacteria. The bacteria were nearly always picked up one by one; rarely were two or more of the microorganisms seen in the same phagocytic pocket. In our previous report (1), we mentioned the possibility that pleural effusion cells were phagocytizing live and dead bacteria alike. In the present experiments, this was clearly not the case. Although there were many dead bacteria in the medium, easily recognized by their homogenous, electron-dense body and their dehiscent cell walls, only structurally well-preserved bacteria were entering HeLa cells.

Once inside the cells, the bacteria multiplied and invaded the cytoplasm (Fig. 5). Bacterial aggregates were most of the time contained in pockets lined by cell membrane (Figures 5-7), but sometimes there was no membrane at all. At first the bacterial growth slowed down, the microorganisms were more loosely distributed (Fig. 6) or even floated free in the pockets (Fig. 8). Multiplication of bacteria was by simple binary fission (Fig. 7).

As demonstrated in Fig. 8, cell multiplication was not impaired by the presence of intracytoplasmic bacteria. Although cell death and destruction were commonly observed in the cultures, the overall vitality of infected HeLa cells remained very good. The cells were easily subcultured by plating the free-floating cells obtained by gentle shaking of the



FIGURES 4a, b, c: Micrographs illustrating active phagocytosis of bacteria by HeLa cells. In 4a and 4b the bacteria are sectioned across, while in 4c a phagocytized bacterium is seen sectioned longitudinally. Note the absence of specialized contact zones between bacteria and plasma membranes. Line: 0.5μm.

culture vessels. Three months after infection, the HeLa cells showed no tendency to slow down or the capability to spontaneously eliminate the bacteria.

Replacement of antibiotic-free medium with medium containing penicillin and streptomycin at somewhat higher concentrations than usual produced rounding of the bacteria, clustering of their DNA filaments, and often dehiscence of their outer walls (Fig. 9). Many were seen in varying stages of disintegration inside and outside the cells or sequestrated in lysosomes, and the number of bacteria-containing HeLa cells was much reduced. Some spheroplasts and filamentous forms were present, but not ''L'' forms. Nevertheless, it was clear that even after 3 weeks of exposure to antibiotics, the HeLa cell culture still remained contaminated by well preserved microorganisms.

Attempts were made to grow the bacteria on media other than those previously tested (1): TSB medium with either 0.1% copper sulfate, 0.1% cupric acetate, or 0.1% cupric chloride. No growth has been observed so far.

DISCUSSION

Inoculation of bacteria into HeLa cell cultures reproduced the same morphological changes as those previously observed in pleural effusion cells (1). The present experiment revealed that HeLa cells phagocytized only live bacteria, which pointed to some active interaction between cells and microorganisms. However, it was clear that while HeLa cells obviously did not need bacteria for their survival, the replication of bacteria was entirely dependent on the presence of cells. In effect, replicating bacteria could be seen in the medium of HeLa cultures, but no replication was observed in the medium alone (1). It must be assumed that the cells contributed to some important metabolic pathway of

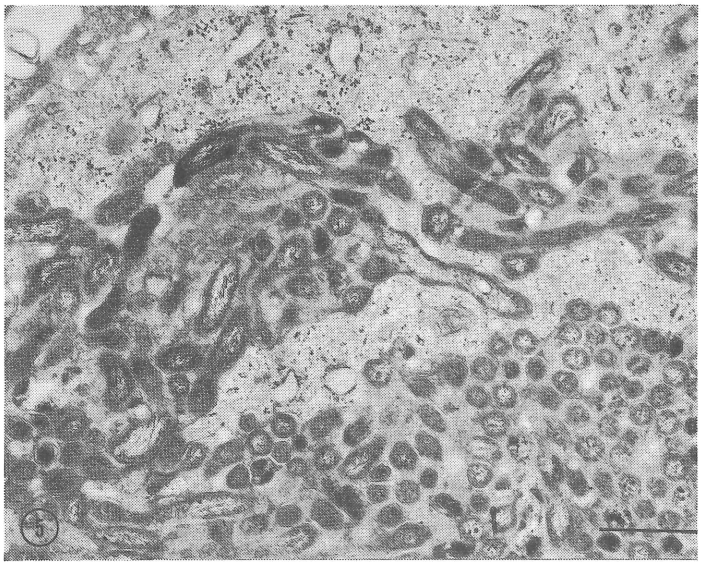


FIGURE 5: Bacteria invading the cytoplasm of a HeLa cell. The microorganisms appear tightly packed in cavities bounded by a continuous cell membrane. Structure of the cytoplasm shows that bacterial invasion is well tolerated by the cell. Line: 1 μm.

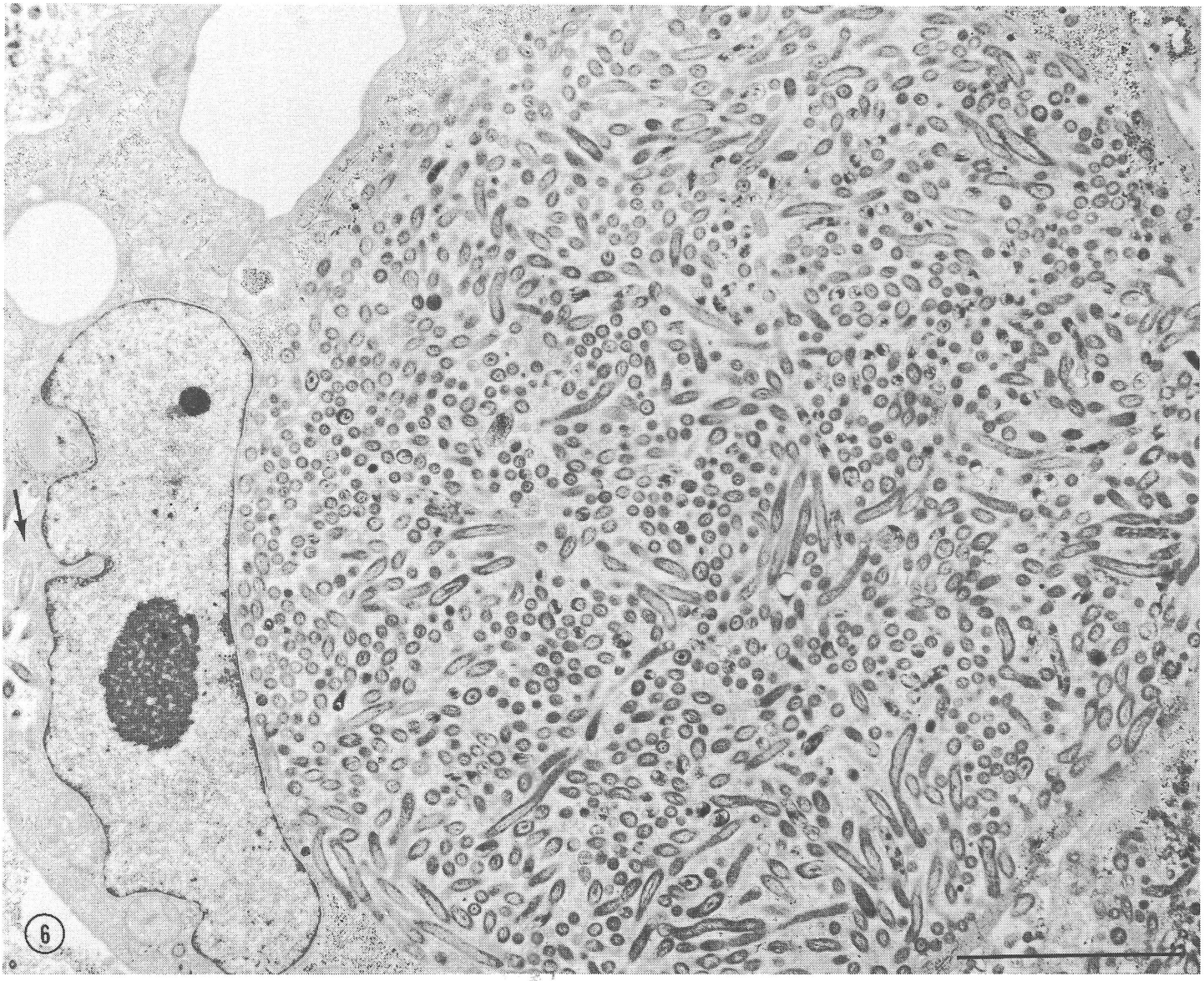


FIGURE 6: Huge bacteria-laden inclusion in a HeLa cell. The bacteria are mostly young; only a few show degenerative changes. The presence of this inclusion does not prevent the HeLa cell from continuing to pick up more bacteria (left side of micrograph, arrow). Line: 4 μm.

the microorganisms, which perhaps are mutants of some saprophytic species and became obligate parasites. Unfortunately, in the absence of an appropriate isolation medium, the species of the bacteria is still a mystery.

Penicillin and streptomycin did severely alter the structure of bacteria contaminating the HeLa cultures. Penicillin is known to interfere with synthesis of the cell wall of gram positive bacteria, while streptomycin is known to inhibit ribosomal functions of both gram-positive and gram-negative bacteria. Which antibiotic caused the structural alterations is not clear. It remains, however, that even at the rather high concentrations of antibiotics used the bacteria were not eradicated. Separate experiments would be needed to determine the right proportions of these antibiotics or to select more efficient antibiotics. For the time being, if further attempts to isolate the microorganisms are fruitless, all the contaminated HeLa cultures will be frozen and stored to be made available for interested investigators.

ABSTRACT

Unidentified gram-negative bacteria contaminating cultures of pleural effusion cells from a breast cancer patient (1) were successfully inoculated into HeLa cell cultures. HeLa cells picked up the bacteria by simple phagocytosis. The microorganisms multiplied in the cytoplasm of the cells, forming large inclusions from which they were released into the medium. Infectious cycle was maintained by bacterial replication both in-

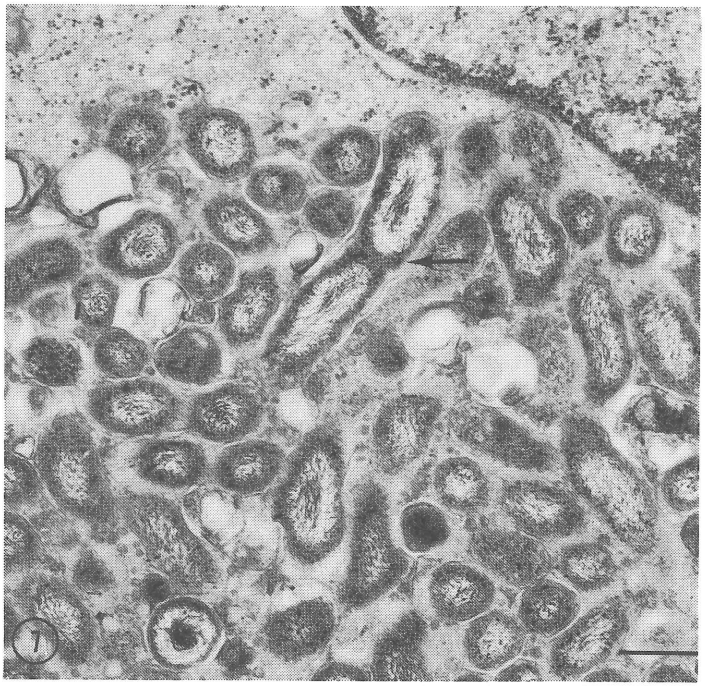


FIGURE 7: Micrograph of intracellular inclusions containing bacteria one (arrow) in a configuration suggestive of binary fission. Line: 1 μm.

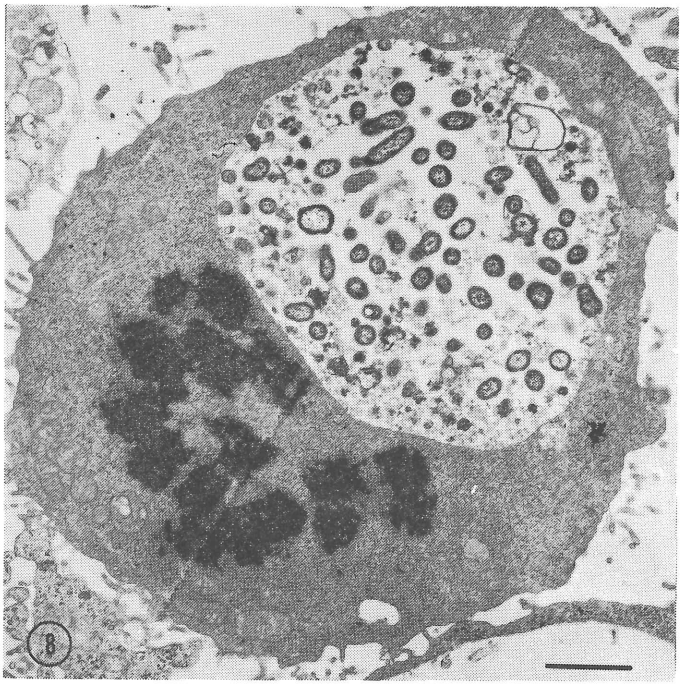


FIGURE 8: Section through a HeLa cell in mitosis containing a vacuole with free-floating bacteria. In this case, the bacteria show degenerative changes. Cell structure remains unaltered. Line: 2 μm.

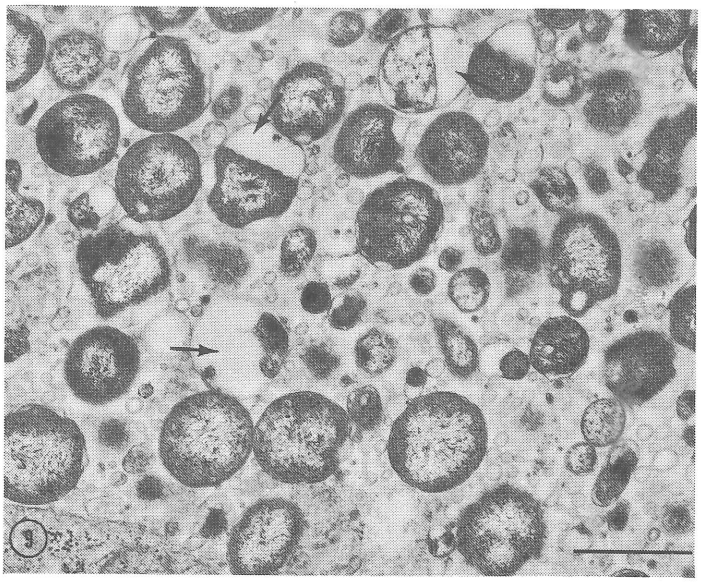


FIGURE 9: Structural changes of bacteria in presence of penicillin and streptomycin added to culture medium. These intracellular microorganisms are rounded and often show lethal damage to their cell walls (arrows). Line: 1 μm.

side and outside the cells. Most infected HeLa cells suffered only scant structural damage and grew well. The bacteria were sensitive to penicillin and streptomycin added to the medium but were not eradicated by the rather high concentrations used. As before (1), the bacteria would not grow on the isolation media tested. Clearly, the presence of cells satisfied some metabolic requirement of these microorganisms, perhaps a mutant strain of a saprophytic species.

ACKNOWLEDGEMENTS

We thank Dr. Roy L. Hopfer and Karen Mills (Department of Laboratory Medicine) for their assistance in bacteriological procedures.

REFERENCES

1. Seman, G. and Cook, C. An unusual case of bacterial contamination in tissue culture: an electron microscopic study. TSEM Journal 13 (3): 5-9, 1982.

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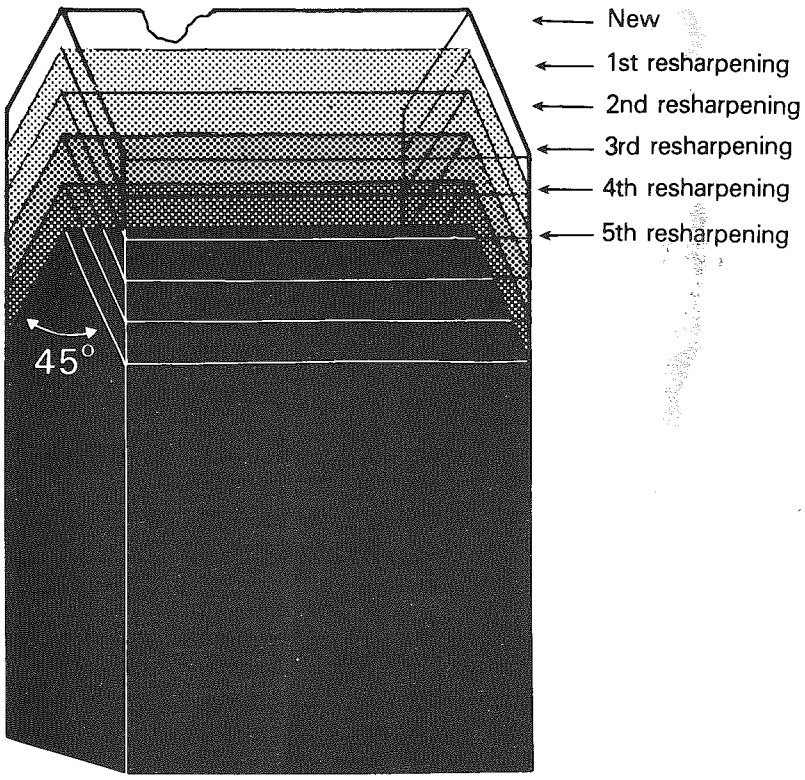

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CASTLES, SCLEREIDS, CRYSTALS, AND THE MICROSCOPY OF SEED COATS

by

M. A. Webb, M.A.
H. J. Arnott, Ph.D.

Biology Department
The University of Texas at Arlington
Arlington, TX 76019-0498, U.S.A.

Research Engineer Scientist Associate I
Dean of Science and Professor

Request reprints from:

Ms. Mary Alice Webb
Biology Department
The University of Texas at Arlington
Arlington, TX 76019-0498

CASTLES, SCLEREIDS, CRYSTALS, AND THE MICROSCOPY OF SEED COATS

Seeds are enclosed in a protective covering, the seed coat, which shields the embryo and nutritive tissues from mechanical damage and provides protection from invasion by pathogens. Two ways in which the seed coat is strengthened against mechanical damage and pathogens are by sclerification and mineralization. However, these modifications also create technical problems for the microscopy of seed coats, primarily because they make sectioning difficult. Scanning electron microscopy (SEM) bypasses these technical difficulties and provides a very effective means for observing and studying the cellular structure of seed coats.

In a volume dealing with plant disease, Horsfall and Cowling (1) suggest that plants, because they are immobile, are in a sense analogous to medieval castles. Plants must, as did the occupants of those castles, employ a variety of defenses against invasion by pathogens. Such defenses begin at the perimeter, in the case of castles, the outer walls and the gates, in the case of plants, the plant surfaces. Plant surfaces may be modified chemically or physically in order to exclude pathogens (2).

In the seeds of higher plants it is the seed coat that forms the outer walls and the gates, the protective barrier against invasion by the "enemy." This protective barrier formed by the seed coat around the softer inner tissues, the embryo and nutrient tissues, is crucial to the survival of seeds. At maturity most seeds become dormant and must survive in this state, often for long periods of time, until conditions are suitable for germination and seedling growth. During prolonged dormancy they are vulnerable to attack by many pathogens, including insects, fungi, and bacteria. Seed coats are modified structurally (3, 4) and/or chemically (5, 6) to provide protection. Mechanical damage to the seed coat can result in significant loss of seeds in storage by providing points of entry for pathogens or insects into the inner tissues (7).

A common structural modification for protection is the formation of sclerenchyma cells, or sclerification, in the seed coat. Sclerenchyma cells are specialized plant cells in which multiple layers of secondary wall material are laid down inside the primary wall. Such cells are generally considered to function in strengthening tissues. Esau (4) described them as "mechanical, or supporting, cells that chiefly lend hardness or rigidity to tissues." The secondary wall thickening may be quite extensive and may in some cases fill virtually the entire cell. Most seed coats have at least one layer of sclerenchyma cells surrounding the seed; many seed coats are composed of several layers of these cells.

Another common way in which plant tissues may be strengthened is by mineralization, particularly by the addition of silica or calcium salts to the cell wall. Haberlandt (8) described measurements of silicified or calcified epidermal walls in terms of their "relative hardness" using a mineralogical method in which hardness was determined by the ability to scratch par-

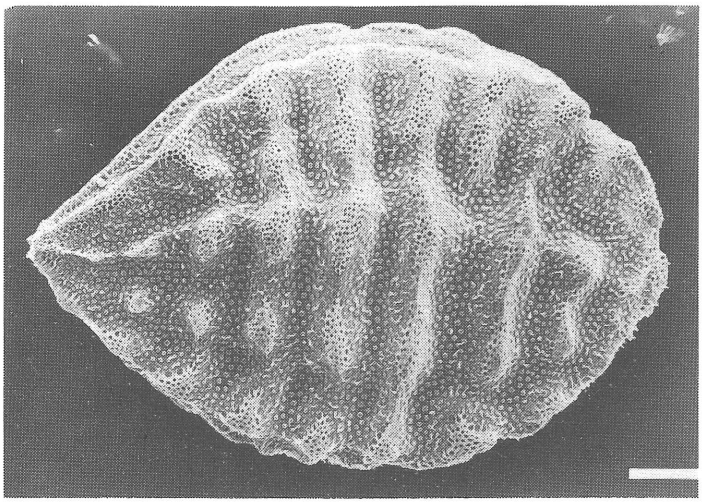

Figure 1. Whole seed of *Oxalis delinii* illustrating surface morphology visible with SEM. Bar = 100 μm.

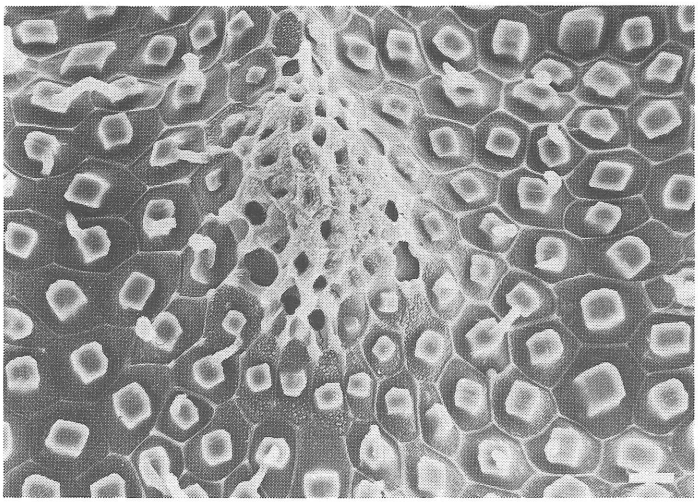

Figure 2. Detail of *Oxalis* seed surface showing crystal cells that form the external layer of the seed coat and a ridge composed of perforated cells. Bar = 10 μm.

ticular minerals. Silicified walls were measured at the highest relative hardness. Calcified cell walls were also relatively "hardened" and often were capable of scratching calcite.

It is not surprising to find that seed coats are also often mineralized. In a survey of mineral deposits in seeds, Webb and Arnott (9) found that the seed coat, or testa, was the most common site for mineralization in seeds. It is extremely common to find an entire layer of the seed coat which differentiates into a "crystal layer," a layer of cells in which each cell is filled with a crystal of calcium oxalate or other mineral substance. Sclerenchyma cells and crystals are quite often found together in the seed coat (9, 10). Several authors have suggested that the presence of crystals may afford added protection (8, 9, 10).

As mentioned previously, the very features that make seed coats effective in protection have also made them difficult to deal with in microscopy. Sclerification and mineralization, either separately or in combination, can cause technical problems in dealing with seed coats when even a relatively small proportion of the seed coat is affected and especially so when much of it is mineralized or sclerified. Because of this, seed coats in general are notoriously difficult to section; for example, Johansen (11) recommended

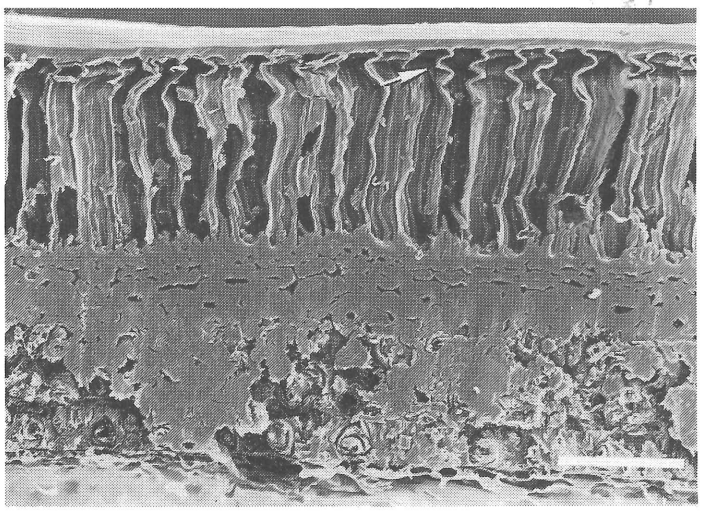

Figure 3. Cross section of watermelon (*Citrullus vulgaris*) seed coat cut with a razor blade. Note artifactual compression (arrow) and tearing of epidermal cells, and "smearing" of structure of other cells. Compare with Figure 4. Bar = 100 μm.

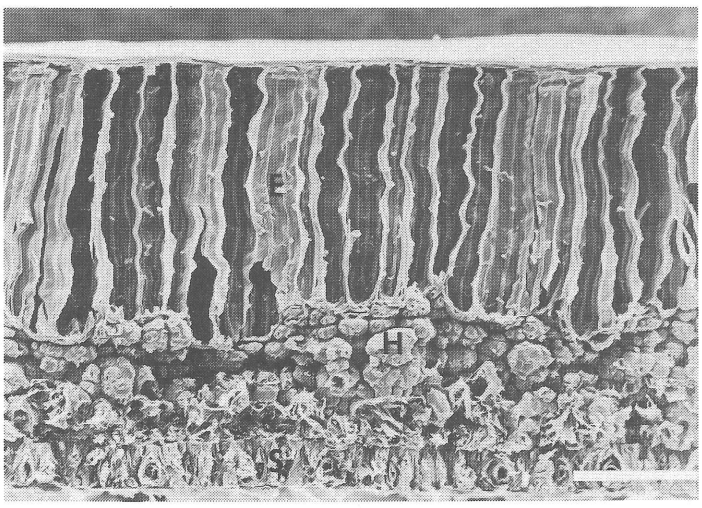

Figure 4. Fractured seed coat of watermelon. Compression of epidermal cells (E) is absent, distinct hypodermal cells (H) are evident. Note the layers of thick-walled sclerenchyma cells (S). Compare with Figure 3. Bar = 100 μm.

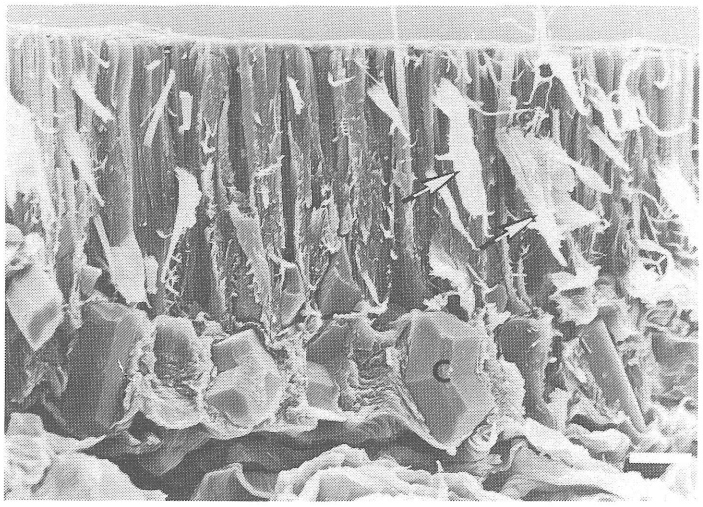

Figure 5. Fractured seed coat of bean (*Phaseolus vulgaris*) showing elongate macrosclereids and crystal-sclereid layer. Fracturing causes tearing of cell wall fibers in macrosclereids (arrows). In the hypodermal layer crystals (C) are exposed. Compare with Figure 6. Bar = 10 μm.

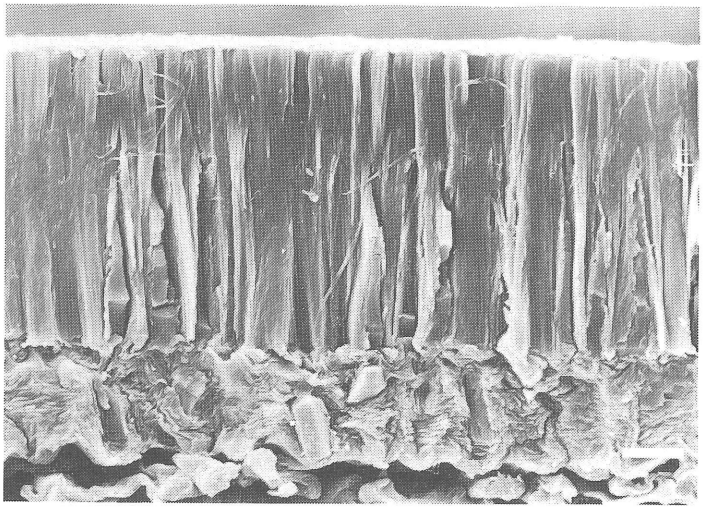

Figure 6. Seed coat of bean which has been cryofractured (fractured while immersed in liquid nitrogen). This technique eliminates artifactual tearing in the macrosclereid layer. However, the fracture plane through the crystal-sclereid layer tends to pass around the cell so that crystals are not often exposed. Compare with Figure 5. Bar = 10 μm.

cutting seed coats into small pieces and treating them as hard wood tissues. Treatment with acids prior to sectioning may soften the tissue, but, of course, such drastic treatment may alter the structure of the cells being studied. Such treatment is particularly undesirable when the structure and distribution of the mineral elements is of primary interest, because it may dissolve any mineral salts present.

SEM provides a means of bypassing the difficulties of sectioning, as well as an extremely effective method for studying the structure of seed coats. The use of SEM can enhance one's ability to observe the diverse ways in which sclerification and/or mineralization occur within seed coats. However, a variety of preparation techniques may be necessary to utilize the SEM to its full potential. Even seemingly minor variations in technique can make a substantial difference in the ability to observe particular features or in the elimination or creation of artifacts.

Since dormant seeds have an inherently low water content, they often require very little preparation for SEM. If one is interested in looking at the seed surface, the seed can simply be mounted on a stub, sputter coated and viewed; countless studies have utilized this method to observe seed surface features for use in taxonomy (12, 13). This simple method is particularly useful when dealing with very small seeds in which even gross surface morphology is not visible by any other means (14, 15). Figures 1 and 2 illustrate the wealth of information that may be gained about surface features of seeds of *Oxalis* in which the surface is composed primarily of crystal cells (16). In O. *delinii*, for example, SEM reveals details such as the size and polygonal shape of surface cells, surface bulges on the outer tangential wall which has collapsed around crystals, papillar extensions of the wall, variation in the size of crystals on a surface ridge, and areas of perforated cells forming the ridges (Fig. 2). According to Netolitzky (10) two outer layers of the seed coat of *Oxalis* are sloughed off during development to expose this crystal layer as the outer layer of the seed.

Seed coats are normally composed of more than one layer of cells, and merely looking at the surface of a seed naturally limits one's knowledge of that outermost layer. Various techniques can be employed to expose the inner cell layers of the seed coat. The simplest involves cutting the seed with a sharp clean razor blade and viewing the cut surface, but in some cases this procedure may result in compression or "smearing" of details. We have found that fracturing or cryofracturing the seed coat usually gives better results for SEM viewing. The normal fracturing procedure involves peeling off the seed coat and bending a piece of it between two pairs of forceps until a break occurs.

A comparison of cut and fractured seed coats of watermelon *(Citrullus vulgaris)* is illustrated in Figures 3 and 4. Note the compression and irregular tearing of epidermal cells and smearing or lack of visible structure in hypodermal cells on the cut surface (Fig. 3). Fracturing generally gives a more artifact free sample (Fig. 4). The epidermal cells are more intact and the individual cells in the hypodermal layer can be recognized. The multiple layering of cell wall material in the protective sclerenchyma layers of the inner seed coat is also clearly visible (Fig. 4).

Simple modifications of the fracture technique may be necessary in dealing with other seeds. In seeds that

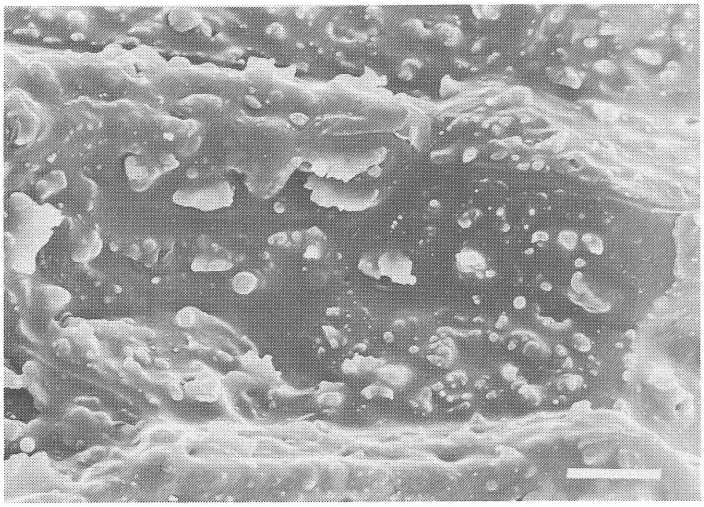

Figure 7. Fractured seed coat of *Magnolia* is composed of oil-filled sclereids. Here the oil obscures other structural details of the cells. Compare with Figure 8. Bar = 10 μm.

Figure 8. Fractured seed coat of *Magnolia* treated with acetone to remove oil. Intricate structural pattern of cell wall layering within the cell is now clearly seen. Crystals are also visible (arrows). Compare with Figure 7. Bar = 10 μm.

are particularly small or hard, a razor blade cut started on one side of the seed can often be induced to fracture through the opposite side of the seed coat. In this way a fractured surface of the seed coat can be obtained.

Seeds of plants belonging to the family Leguminosae are known for having very hard seed coats, and sclerification plays a prominent role in the structure of such seed coats. In bean *(Phaseolus vulgaris)* seed the epidermal layer of the seed coat is formed by very elongated sclerified cells, termed macrosclereids or palisade cells (3, 4). In a simple fracture a great deal of tearing and displacement of cell wall fibers normally occurs (Fig. 5). For cleaner fractures the piece of tissue can be broken while immersed in liquid nitrogen, or "cryofractured" (17). In the bean seed this eliminates artifacts associated with tearing of the cells, and for the most part the fracture surface is more regular (Fig. 6). However, one point that cannot be stressed too greatly is the fact that as in any microscopy, the technique used must be determined by the structures of interest to the investigator. An example of this is the different appearance of the subepidermal layer in *Phaseolus* (Fig. 5 and 6). This layer is composed of crystal-sclereid cells and in the simple fractured seed coat, the fractures tend to break through the cells and around the crystals, leaving them exposed (Fig. 5).
In cryofractured tissue (Fig. 6) the fracture plane follows a different path, and crystals are most often not exposed. The simpler fracture technique is obviously preferable if the crystal structure is a primary interest. The structural barrier created by combined sclerification and mineralization is clear in both cases.

Another problem frequently encountered in observing seed coats with SEM is the presence of oil within the cells. This is true for sclereid cells in the seed coat of *Magnolia* shown in Figures 7 and 8 (seeds collected in Galveston at the Fall 1982 TSEM Meeting). In the SEM unfixed oil tends to cover surfaces and obfuscate details of the other structural components. The oil characteristically has a smooth "fluid" appearance with irregular globules present where it covers other structures (Fig. 7). The simplest way to deal with this oil is to remove it with acetone or other lipid solvent in order to see the other cellular structures (18). Figure 8 illustrates a cell in the *Magnolia* seed coat which was treated by washing with acetone. It illustrates not only the extremely interesting structure of the crystal-sclereids revealed by this procedure, but also the intricate pattern of cell wall "swirls" and the layering that forms the internal structure of the cells. The *Magnolia* seed coat is composed of several layers of these unusual cells. Sclereid cells in the seed coat of *Vitis vinifera* and *Ricinus communis* viewed with SEM have a similar structure of concentric layering surrounding numerous pores (Webb, unpublished).

In some cases tangential views of cells within the seed coat may be useful for observing structural details not visible in sections. Such views can be exposed by simply peeling off successive layers of cells until the layer of interest is exposed. In some instances the outer layers can be broken off the dry seed; in other cases hydration of the seed facilitates peeling off the layers. In papaya (*Carica papaya)*seed a crystal layer which occurs in the seed coat can be exposed by this means to show the large crystals which almost fill the cells (Fig. 9). These crystals have characteristics consistent with a chemical composition of calcium oxalate (9), and may present a chemical as well as physical barrier to penetration of

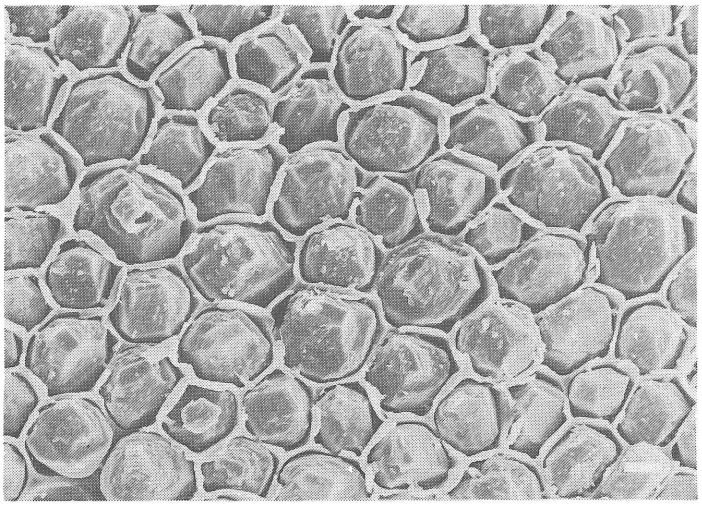

Figure 9. Tangential view of crystal layer in the seed coat of *Carica papaya* shows crystals which almost completely fill the cells and illustrates the barrier this layer presents to penetration of the seed. Bar = 10 μm.

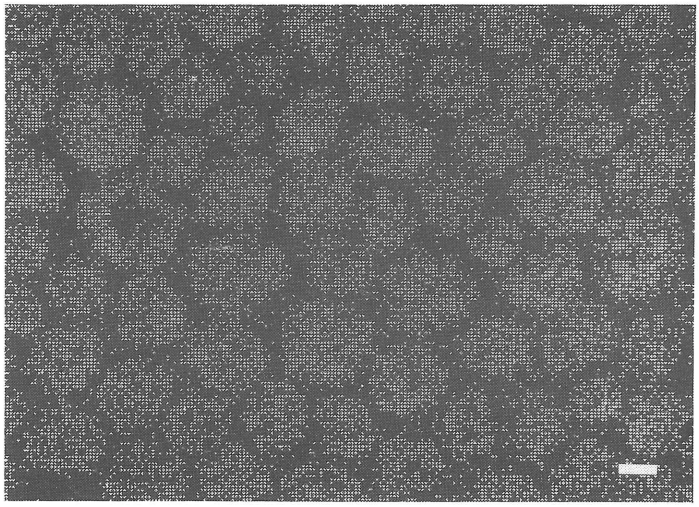

Figure 10. EDX analysis of crystal layer in papaya seed shown in Figure 9 mapping the distribution of calcium, which may pose a chemical as well as a structural barrier to pathogens. Bar = 10 μm.

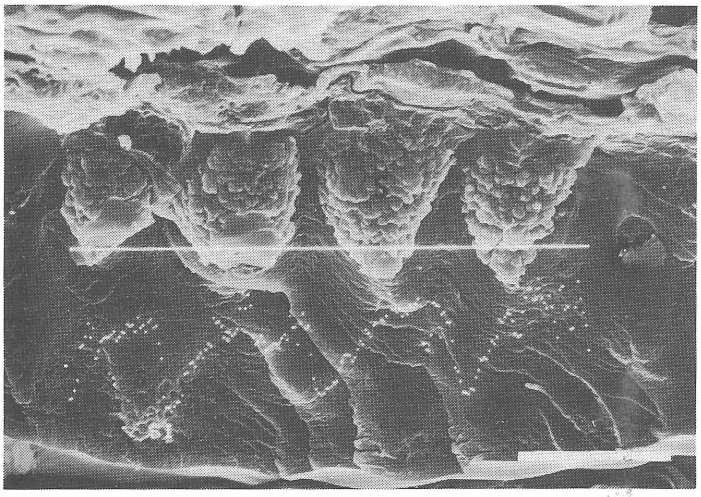

Figure 11. A silica-sclereid layer in the seed coat of cardamom (*Elettaria cardamomum*) seed in which silica bodies (S) fill up one end of the cells, the remainder of which are completely sclerified. The figure illustrates a linescan for silica content, the upper line showing where the specimen was scanned and the lower line representing the silica content. Bar = 10 μm.

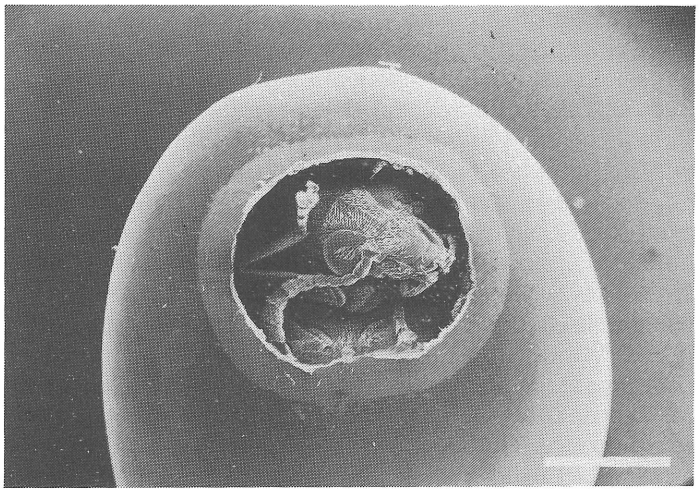

Figure 12. A bruchid beetle inside a seed of *Cercis canadensis* showing the circular hole it has cut in the seed coat for exit. The beetle has eaten out the inner seed tissues. Bar = 1 μm.

this seed. Figure 10 maps the distribution of calcium over a portion of such a layer, which forms an almost continuous "calcium barrier." Such a barrier presents special problems to any organism trying to penetrate the seed coat. In this case a tangential view of this crystal layer best illustrates its protective structure.

In seeds of members of certain other plant families a similar barrier is formed by the characteristic deposition of silica in the seed coat (9). Such is the case in cardamom (*Elettaria cardamom*) seed in which spherical silica bodies fill one end of the sclerenchyma cells surrounding the seed (Fig. 11). As illustrated here, SEM in combination with EDX analysis is an especially useful tool for identifying the particular type of mineralization present in a seed coat and has enormous utility in the ability to precisely locate mineralization within the cells. There is some evidence to suggest that silica content in plants is in-

versely related to infection by fungi or other pathogens (19), so its characteristic presence in the seed coat of certain seeds (10) can be considered to have significance for protection.

Though they may be designed for protection, seed coats, like castles, are not entirely impenetrable, and some very interesting strategies have evolved among pathogens that penetrate seeds. Primary insect pests of seeds are able to gain entry into mature seeds by boring holes in the seed coat (7). The insects then feed on the internal seed tissues, leaving a hollowed-out, empty shell, the seed coat. Other insects not able to penetrate the coat must rely on mechanical damage, often caused by harvesting, as a means of entry. Many insects adapted to feeding on seeds actually enter the young developing seed when seed coat tissues are immature and still soft. The adult bruchid beetle, for example, oviposits into the legume at such an early stage of development. After the eggs hatch, the larvae develop inside the seeds and feed on the developing seed tissues. By the time the beetle matures the seed coat is also mature and very hard and so the beetle must penetrate the seed coat in order to escape. It accomplishes this by actually cutting a circular disc in the seed coat through which it can exit (20). In collections of seeds, circular holes in the seed coat are seen, as well as the corresponding circular discs of seed coat that have been cut out. This technique obviously involves dealing with a minimal amount of seed coat in order to get the proper sized exit hole. Figure 12 illustrates a *Cercis canadensis* seed coat in which a beetle has cut such a hole, showing the torn macrosclereids on the cut surface. The beetle's mandibles, which are modified into wedge shapes for this cutting, are folded out of sight in this view.

In summary, sclerification is well-accepted as a means of strengthening plant organs, but a similar role for mineralization in plants is less well-established. The structural or skeletal role of mineralization is familiar to all of us, for example, in the formation of shells or bones in animals and/or in microorganisms, as in the valves of diatoms. A good case can be made for a skeletal role of mineralization in seeds. The prominence of mineralized tissues in the seed coat, particularly in association with sclerification, increases the hardness and thereby enhances the protective capacity of these "outer walls" surrounding the plant embryo. With careful attention to techniques scanning electron microscopy can contribute greatly to our ability to observe and study the diverse manners in which both sclerification and mineralization occur in the seed coat.

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SV: CALCULATION OF THE SURFACE TO VOLUME RATIO IN BIOLOGICAL MATERIAL

by

Wayne R. Fagerberg, Ph. D.

Department of Biology
Southern Methodist University
Dallas, Texas 75275 U.S.A.

Request reprints from:

Wayne R. Fagerberg, Ph.D.
Department of Biology
Southern Methodist University
Dallas, Texas 75275

Since Weibel popularized quantitative electron micrograph analytical techniques in 1972 stereology has slowly become recognized as an essential tool in the description of cell structure. The advantages of these techniques are that they remove a major source of error in interpreting data (investigator bias), allow detection of relatively small changes in structure that would remain undetected with other techniques, provide reliable data that can be evaluated statistically, (incorporating error statements in the data interpretation), provide a practical method for comparing results of investigations by separate labs, and provide a mechanism by which structure can be correlated to biochemical and physiological data.

Although the mathematics in stereology appears complex, it is straight forward addition, subtraction, multiplication and division; in fact it is so simple it is hard to believe that it works. The mathematical foundations of the techniques were established over 140 years ago and their validity has been proven over and over again since that time (see DeHoff and Rhines, 1969; Underwood, 1970; Weibel, 1979). Although the mathematical foundation of the technique has been established and the derivation of data from micrographs is relatively straight forward there are some problems of application that must be addressed. Hopefully this paper will help alleviate some of the questions about deriving the S_V (surface to volume) parameter from micrographs with a "how to" approach and a discussion of some of the common problems to watch for. Finally, some aspects of statistical handling of the data will be discussed. As confusing as the calculation of the S_V ratio may appear at first glance once one begins to use the technique its simplicity will emerge.

What is S_V

Basic to understanding this measurement is an understanding of stereology in general. For a detailed description of this the reader is referred to other sources (Underwood, 1970; Weibel and Bolender, 1973; Fagerberg and Arnott, 1977; Weibel, 1979). Suffice it to say here that information for this parameter must be obtained from a number of samples (usually a micrograph = one sample, discussed in a later section) and data from the various samples integrated together to yield a "mean" or average value. In other words, we describe a value that is representative of a population of cells/organelles rather than a single cell and describe this population in terms of an average cell in that population. Also, except under special circumstances, each sample must come from a separate cell in that population (i.e. no serial or two sections from the same cell).

The S_V ratio describes the relationship between the two dimensional surface area of a structure (hereafter called the α structure, e.g. membrane) per unit three dimensional volume of the containing structure (called the β structure, e.g. cell, chloroplast, mitochondria, etc.). It is important to realize the surface area and the β volume are described in the same units of measure (e.g. μm, A, mm, etc.). Since the S_V value is a ratio it states that per cubic unit of volume of the β structure there will be x^2 units of α structure surface area. It does not make any statement as to the distribution of the surface area within the β volume or the total amount of α in β.

Calculation of S_V

Although several mathematical formulae have been presented for the calculation of the S_V ratio they all basically rely on defining the size of β by a system of test lines or a combination of coherent points and lines that randomly intersect the β profile on a micrograph (Weibel, 1979). As an example one might describe the S_V ratio of chloroplast membranes in the chloroplast. In this case the chloroplast membrane is the α structure and the chloroplast profile is the β structure. The test lines to be measured or counted are only those that fall within the chloroplast profile boundary (Fig. 1). However, one might also be concerned with the S_V ratio of chloroplast membranes per unit volume of the cell. In this case all the test lines within the cell profile are to be measured. In this discussion we will use the random line test system to calculate S_V as it tends to be less subject to bias than the coherent point methods. The mathematical relationship which defines the S_V ratio is:

$$S_V = 2P_L \text{ unit}^{2}/\text{ unit}^{3} \quad \text{(Underwood, 1970)}$$

where P is the number of intersections between the test lines and the α structure whose surface area is being measured and L is the total line length of the test lines that lie within the β profile.
The units are determined by the units used to measure the test line length (more on this later). It is important that the line length be accurately measured and that the test system of lines adequately sample the entire β profile. Thus it is important that both the boundary of β and the α structure are clearly definable on the micrograph. Also, **it is essential** that the test lines randomly intersect the α structure if the relationship described in the mathematical formula is to be valid. This becomes especially critical when the α structure is highly ordered within β (e.g. thylakoids in chloroplasts, more on this later). It is also important that the organelle upon which S_V measurements are made must be randomly selected. That is, without investigator bias, for example an apriori determination that every other organelle will be measured or every organelle under a random point grid thrown onto the sample micrograph, etc.

Example-Measurements

A) S_V ratio of thylakoids in chloroplasts:
A random line transparency (thermofax transparency, see Fagerberg, 1980 for construction) is placed or thrown onto the β profile and the following measurements made: (Figure 1)

1. total line length (L) = 606 mm
2. # of intersections with α = (P) = 32

therefore

3. $S_V = 2 \cdot \frac{22}{606} = .11 \text{ mm}^2/\text{mm}^3$

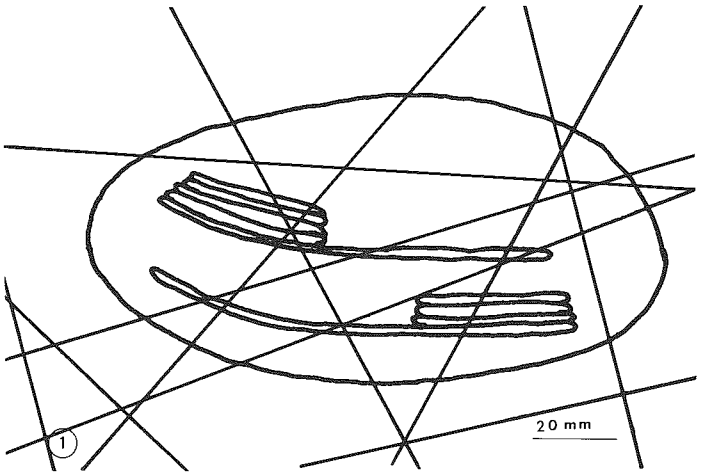

Figure 1: S_V ratio of internal membranes using random line test system applied to model membrane system. Line length (1) is the total sum of line lengths within the boundary of the oval. A 20 mm reference line is provided to calibrate changes in magnification which occur during printing.

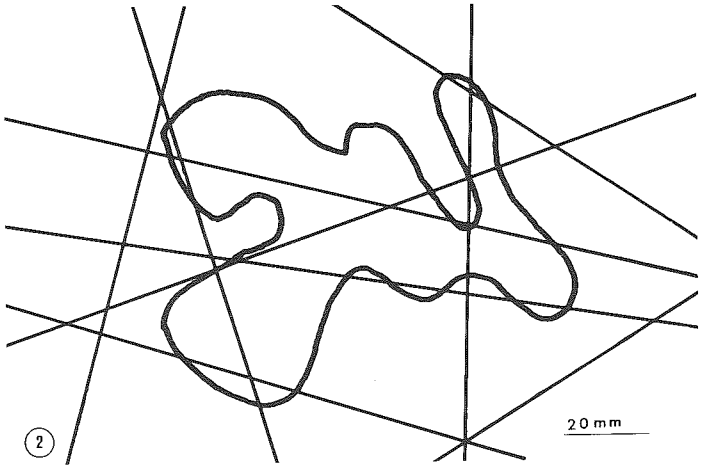

Figure 2: S_V ratio of boundary membranes using random line test system applied to model profile. Line length (1) is the sum of all lines within the profile boundary. Note, each straight line segment will generate two points of intersection (P) with the profile boundary.

B) S_V ratio of boundary membrane (Figure 2)

1. total line length (L) = 291 mm
2. # of intersections of lines with α = (P) = 26

therefore

3. $S_V = 2 \cdot \frac{26}{291} = .18 \text{ mm}^2/\text{mm}^3$

It can be observed in example A that if test lines do not randomly intersect the highly ordered chloroplast membranes inaccurate S_V ratio would result. In the first example we used a random line grid, if there

were questions of biased sampling we could rotate the test grid, throw it down on the micrograph and make measurements again averaging the two S_V ratios that result. Another alternative is to construct a curvilinear grid (Weibel, 1979 after Merz).

C) S_V ratio using curvilinear grid Figure 3.
- in order to simplify the measurement of total line length we use only those half circles that fall entirely within the β profile. (asterisk)
- Line length is calculated;

$$L = P_t\,(\pi/2)\cdot d \qquad \text{(Weibel, 1979).}$$

therefore

$$L = 8\cdot(\pi/2)\cdot 29 = 364$$

where P_t is the number of half circles within the β profile (modified from Weibel by WRF), and d is the diameter of the half circles in mm or appropriate units. Once line length is determined then calculation proceeds as previously described. In this case S_V = .11 mm^2/mm^3

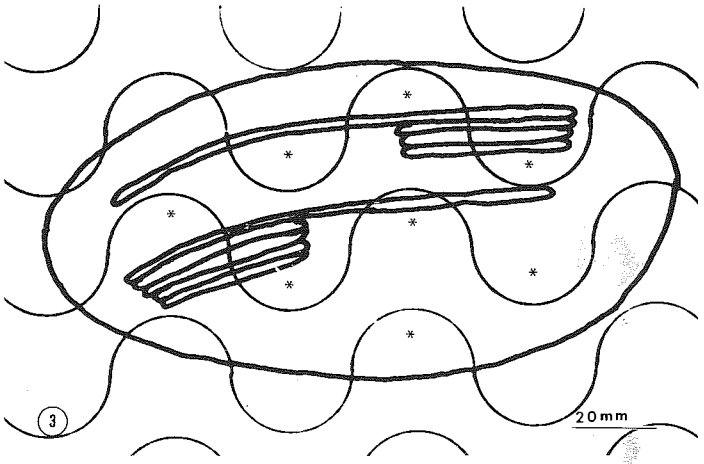

Figure 3: S_V ratio of internal membrane system using curvilinear test line system. Line length (1) is calculated from only the half circles totally within the boundary of the profile (asterisk). Line intersections are scored only on the half circles used to calculate total line length.

Since the S_V data must be taken from a number of micrographs to be representative of the 3-D β structure the S_V ratios will be a mean value. There are two methods by which $S_{\overline{V}}$ (mean S_V) can be derived, which method to use depends on the nature of β (see Mayhew and Cruz-Orive, 1974; Weibel, 1979) but in general if the size of the β profile changes from one sample to the next method #2 should be used.

Method 1

$$S_{\overline{V}} = \frac{\Sigma_o^n S_V}{n} \text{ or } 1/n\cdot\Sigma_o^n S_V$$

where n is the number of samples and S_V is value obtained in each sample (e.g. all organelles in one micrograph, more on samples later).

Method 2

$$S_{\overline{V}} = 2\cdot\frac{\Sigma_o^n P}{\Sigma_o^n L}$$

(Weibel, 1979, mod. from Mayhew and Cruz-Orive, 1974). Where P and L have been described previously.

In method 2 one sums all of the P values and divides that number by the sum of total line lengths L. Based on sampling problems described by Mayhew and Cruz-Orive (1974) method two should be used when the size of the β profile is not consistent from one micrograph to the next. Thus the second method is more proper in most circumstances. However, it results in only a single S_V value rather than being derived from a population of S_V values as in method 1. Therefore one can't describe normal distribution by common statistical procedures. Special statistics will be described later to overcome this problem.

Correction For Magnification

Most S_V values will be calculated from micrographs that are enlargements of the real object. Any change in magnification, if left uncorrected, will have a great effect on the S_V value. It may also be desirable to correct for changes of the micrograph which occurs during processing since these will have the same effect on S_V values as changing the magnification. Generally it is easiest to make line length measurements from the micrograph in mm since these can easily be converted to other units. In correcting for magnification one can also convert mm to other units at the same time. Magnification correction is made by correcting for true or actual test line length (L_C in the following formulae) in terms of the actual size of the β structure.

Assuming measurements on the micrograph were made in mm correction for actual test line length is as follows:

1. For mm, $L_C = \frac{L}{mag}$
2. For μm, $L_C = \frac{L}{mag}.x10^{-3}$
3. For A, $L_C = \frac{L}{mag}.x10^{-7}$

Where L_C is the total line length corrected for magnification (Mag.) and L is the total line length (in mm) applied to the micrograph (s). The units for S_V from these three equations would be mm^2/mm^3, $\mu m^2/\mu m^3$ and A^2/A^3, respectively.

In our lab we usually convert all S_V values to μm. The calculation for S_V now becomes:

$$S_{\overline{V}_C} = \frac{\Sigma_o^n P}{\Sigma_o^n L_C} \text{ units of } c^2/\text{units of } c^3.$$

(e.g. using data from Fig. 1 and a print mag. of 20,000 - S_{V_C} = 2.11 $\mu m^2/\mu m^3$)

if correcting for print changes becomes important then shrinkage/expansion factors must be determined by a system of standard lines projected onto sample prints. Once the percentage change from the standard line length is determined L_C must be increased by that percentage if shrinkage occurred and decreased by that percentage if expansion occurred. In most cases these changes are relatively small and to make matters more complicated not equal in all directions. Since all micrographs, presumably, will be equally affected we generally ignore them but when calculating real or actual surface areas (later sections) we must realize that there will be a small error introduced. This problem can be overcome by obtaining measurements directly from projections of negatives.

Statistics

One of the advantages to describing cell structure in quantitative terms is that one can define levels of confidence about the data. In many cases this simply involves summing the mean ratios from a series of micrographs (samples) and proceeding with normal statistical tests (note: it may be necessary to carry ratio data through arcsine transformations before doing this, see Sokal and Rohlf, 1981).

In addition to the mean, variance is a most important basic statistical parameter since other distribution parameters (e.g., std. dev., S.E., Confidence intervals) and test of significant differences among means are based on it. We will consider two methods for calculating variance. In one case we can divide our micrographs into "representative samples" groups (Weibel, 1979), calculate a mean for each group and carry out normal statistical test on the means from each group. However, recall that if the β profile size changes from one sample to another it is more accurate to calculate the mean S_V from the total sum of P and L_C. Since this mean value is not derived from a population of values normal statistical procedures can't be used. Cochran (1953) developed a series of procedures to deal with ratio estimates such as this. Both methods are briefly outlined later.

Before proceding with statistical test we must answer the question, "what represents a single sample," one micrograph/organelle, two micrographs/organelles? Although no clearly satisfactory method of answering this question has been presented, Weibel (1979) has proposed a method for calculating a "representative sample" that is used to generate a single sample. A number (n) of representative samples are then summed together to yield a statistical population. The representative sample is a conservative estimate and one can achieve quite satisfactory results with less effort but the idea is presented here as a method of approaching the problem of sample size. Representative sample size for S_V ratios is defined in terms of the total line length of the test system that must be measured for a single representative sample. The number of micrographs that must be measured to achieve this line length is determined and all the data from those micrographs are summed together for one S_V value. The next group of similar number of micrographs is handled the same way and so on.

Total line length for one representative sample:

$$L_t = \frac{4}{S_V \cdot RSE^2\ (S_V)}$$ (Weibel, 1979, p. 118)

where L_t is the line length applied to one representative sample, S_V is an estimate derived from analysis of several micrographs/organelles and RSE (S_V) is the percent error (in decimal) the investigator is willing to accept (e.g., 10, 15, or 20%).

Example: $S_{V_C} = 3.4\ \mu m^2/\mu m^3$

RSE (S_V) = 10% then

$$L_t = \frac{4}{3.4.\ (.1)^2} = 117.65\ \mu m \text{ of line length}$$

Since the line length is in terms of actual length it must be converted to the length in mm that must be measured on the micrograph:

$$L_1 = \frac{\text{Print Mag.}}{1000\ X\ L_t} \text{ in } \mu m$$ where L_1 is the total line length in mm that must be applied to the micrograph (note: this relationship is valid only if you use μm as real line lengths, A and mm require different formulae). If print mag. = 20,000 then 2350 mm of line in β profile must be measured in each representative sample. This effort could be reduced if larger percent errors are accepted.

If normal statistical procedures are used then S_V ratios from each representative sample are summed and the standard distribution statistics described (e.g. mean, std. err., std. dev., variance; Sokal and Rohlf, 1981). A second method of describing statistical parameters for ratio estimates has been modified from Cochran (1953) by Weibel (1979). These methods are the only ones that can be used if S_V is calculated by the formula $S_{V_C} = 2 \cdot \frac{\Sigma P}{\Sigma L_{t_C}}$ since all data is summed to result in a single mean. One can still sample the same number of micrographs as determined previously but in this instance they will all be lumped into a single mean value.

Calculations of variance SD^2 (S_V)(Weibel, 1979):

A) $R = \frac{\Sigma_0^n P}{\Sigma_0^n P\ L_{t_C}}$ where R is the basic ratio

then $S_V = K \cdot R$ where K is a constant (in the case of S_V it is 2).

B) $SD^2(R) = \frac{n}{(n\text{-}l) \cdot (L_{t_C})2} \quad (\Sigma P2 + R\Sigma L_{t_C}{}^2 - R\Sigma P \cdot L_{t_C})$

then $SD^2(S_{\overline{V}}) = K^2 \cdot SD^2(R)$
and Standard Deviation =
$SD(S_{\overline{V}}) = \sqrt{SD^2(S_V)}$
and Standard Error =
$\text{Std. Err}(S_{\overline{V}}) = \frac{SD(S_{\overline{V}})}{\sqrt{n}}$

Discrimination between the validity or "strength" of these two methods of calculating distribution parameters must be left to more mathematical minds. However, the second method described here is more conservative and therefore more "powerful". Currently both methods should be acceptable but the investigator should make known which was used.

The classical t statistic used in the majority of biological literature may or may not be used in stereological work depending on the validity of several assumptions (Sokal and Rohlf, 1981). If these cannot be met or if one is not sure there are a number of non-parameteric (distribution independent) statistics that are useful. Although these generally are not considered to be as discriminating (conserative) as distribution dependent statistics there is no reason to ignore them as they are valid and just ways of handling data.

Correction For Section Thickness

Stereological methods are based on the assumption that profiles will be measured from true 2-D surfaces. When measurements are made from micrographs derived from sections, no matter how thin, they represent projections of 3-D volume onto a 2-D surface. Depending on the size of the α structure (e.g. membranes) and the section thickness this can cause considerable error in calculations (see Holmes effect, Weibel, 1979) often necessitating correction factors. We can generally ignore these correction factors (and other correction factors except magnification) in two situations:

1. If the diameter of the α structure is 10 times or more the thickness of the section.
2. If we are interested in relative comparisons between two experimental groups in which the realtionship between α diameter and section thickness does not vary. However, without correction other labs or later work may not be directly comparable to the original work.

If one desires to very accurately calculate actual surface area within the β structure then correction becomes more critical. Exactly how serious the error generated by failure to correct for these effects has not been conclusively worked out at present. This is one area of stereological methodology where we should expect to see new methods appearing. However, for the present Weibel (1979) has described several methods for section thickness correction depending on the α geometric shape which might help correct inherent measuring errors. Before applying these correction factors one must determine the geometric shape of the surface area being measured (e.g. does it form a disk, sphere, or cylinder), then whether there is a problem of overlap of structures within the sections, and the section thickness. Once a series of measurements (diameters-lengths) have been made and averaged the calculation of the correction factor from graphs (p. 139, Weibel, 1979) is relatively straight forward.

Section Compression

Another problem that can be of some importance in calculating the most accurate actual surface areas within cells is the effect of section compression. Again, this is not as important if one is simply making relative comparisons between two experiments as both should be affected equally. The amount of compression depends on many factors, plastic hardness (harder is better), section thickness (thicker is better), knife sharpness, knife angle, and specimen hardness. Compression is usually a directional problem with the majority of distortion along one axis, therefore line lengths measured along that axis need to be corrected while those in other axis will not. This makes correction more complicated. The formula for compression correction is given in Weibel (1979) and involves measuring the object (e.g. block face) before sectioning and after sectioning (e.g. section length) and working through the relationships given. How critical these measurements are has not been established and probably should be dealt with by each lab in each experimental situation.

Effect of Resolution

A final problem to deal with is perhaps the most intriguing. It has its basis in the calculation of "Fractal dimensions" first described by Mandelbrot (1977) which shows that the more accurately you measure a curved line the longer will be its length-to a point. As an example if one tries to measure the coast line of Britain using areal photographs the length would be considerably less than someone walking the coastline with a measuring wheel, carefully measuring all the ragged edges between water and land. If one were to train an ant to measure the coastline of Britain, carefully measuring the boundary of each sandgrain it is easy to imagine the dimensions would be infinite. How closely should you look or what is the real coastline of Britain? The question relates to membranes in the same way, as resolution increases the ability to measure small irregularities increases. This is an important problem for all quantitative type measurements. Thus if one person were to measure an S_V at magnification X and a second person at magnification $X \cdot 3$ the second could record a different value. This creates a problem in trying to com-

pare work from different labs if conditions of measurement were not exactly the same. In order to deal with these irregularities, Paumgartner et. al (1981) have proposed that we standardize all our measurements. Based on the idea that there is a convenient magnification (one which is the least tedious and still provides sufficient accuracy to make good S_V measurements) and a magnification where any further increase in magnification does not increase the S_V values (the latter is usually much too high to be convenient). Utilizing these two magnifications one can calibrate a standardization factor which will allow measurements at the convenient magnification to have the values of those made at maximum information magnification. These standardization calculations probably need to be made for each experimental situation and are relatively simple. By taking a series of photographs at increasing magnifications of a typical α structure one can determine at which mangnification the S_V no longer changes (max. mag.). Knowing this and the magnification at which it is most convenient to measure the $S_V$ratio a standardization factor can be plotted (see Weibel, 1979). This is an important parameter because it will aid in standardizing S_V values between labs and allow better, more accurate determination of S_V.

Essentials of resolution correction factor:

1. plot log/log graph of S_V at different magnifications.
2. log of low (convenient) mag - log of high mag. $= m\Delta$
3. log of low S_V - log S_V high value $= P\Delta$
4. then slope $= P\Delta \div m\Delta$
5. correction factor cfm = (mc/me) slope
   where mc = critical maximum mag.
   me = efficient mag.
6. then, cfm $\cdot S_{\overline{V}} = S_{\overline{V}}$ corrected.

Actual Surface Area

Ratio values are important for describing the relationship between surface density and volume but this relationship has limited information value. Ratio values may even be misleading in some instances when similar ratio values occur in organelles of dissimilar size (or vice versa) leading one to the conclusion that a similar amount of surface area was present in both cases. Depending on the experimental question it may be prudent to convert dimensionless ratio values to values with dimensions (i.e. actual mm^2, μm^2, etc.). This provides a description of the actual amount of surface present per mean cell and by determining the number of cells present in a tissue or organ the total amount of surface area present in the whole structure may be described. This information may be very useful in correlative biochemical or physiological studies.

Calculation of the actual surface area is relatively simple but a few precautionary statements should be made. First, actual surface area per mean cell is calculated by determining the actual volume of the β structure per mean cell and then simply multiplying that number by the S_V ratio value. It is important that the β volume be described in the same units of measure as the S_V ratio. If β is an organelle then it may be easier to first calculate the percent volume (V_V, Underwood, 1970; Weibel, 1979) that organelle occupies in a mean cell. Then the actual volume of the mean cell is determined and the β V_V value is multiplied by that number resulting in the actual volume of the β per mean cell. Since it is difficult to accurately describe the volume of a single cell or organelle we must often use geometric representations of these structures, then by making appropriate measurements from a number of micrographs determine the proper mean proportions that will allow calculation of volume based on the presupposed geometric model. Obviously this is going to introduce an additional source of error, the error being greater the greater the deviation of the cell from the ideal geometric model being used to describe it and our ability to measure the model defining parameters. This error can create problems but if the cell can be modeled sufficiently well the estimated actual β volume will be reasonable. Also, if the mean β volume between two samples can be shown to be statistically different then the resulting actual surface areas will be considered different even if the ratios were the same. If cells cannot be described using geometric models it may be possible to work backwards to obtain mean cell volume. An example: usually cell nuclei have a reasonable definable shape, if one determines a V_V value for the nucleus then measures the mean nuclear diameter (if the nucleus is spherical or if disk shaped) and thus determine the nuclear volume. The cell volume may then be determined by $1/V_V$ (in decimal) X actual mean nuclear volume = cell volume. This method adds another level of error to the final surface estimate but in most cases still provides a more accurate actual surface area estimate than any other technique. The most critical factor is understanding the 3-dimensional structure of the β structure so that an accurate geometric model may be ascribed to it and accurate and appropriate measurements made from sections.

Although all of this may seem rather complicated to those who haven't attempted these calculations, working through several sample S_V problems will help establish an order to the measurement procedure. Once such a procedural order is established the ease of the calculations will become evident, the math is all straight forward and easily handled by "pocket" calculators. The decisions concerning correction factors is not as clear and the reader should refer to other texts for better understanding of these. The application of stereology to biological material will continue to see procedural improvement, and we must continually be aware of these new improvements as they occur.

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(Continued From Page 3)

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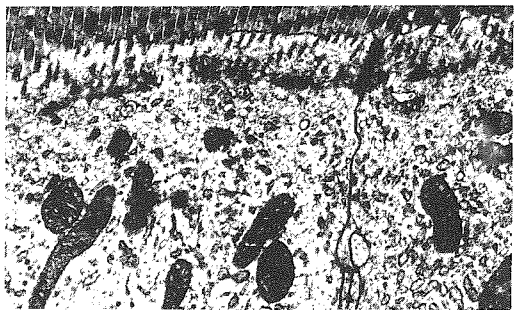
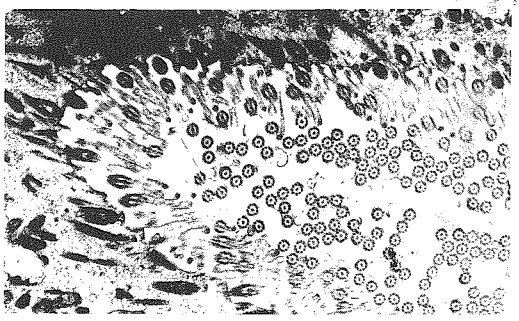


Hamster intestinal epithelium



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Dodson, R.F., Williams, M.G., Jr., and Hurst, G.A.: Method for removing the ferruginous coating from asbestos bodies. J. Toxicol/Environ. Health: In Press.

NEW FACULTY AND/OR STAFF

Hitoshi Maeda, M.D., a visiting Clinical pathologist from Kobe University's School of Medicine, Kobe, Japan, has joined the Department as a Postdoctoral Research Associate.

Jan Lewandowski, M.D., Ph.D., with specialities in Obstetrics/Gynecology and Biochemistry, has joined the Department as a Research Scientist.

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NEW FACULTY

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PUBLICATIONS

K. Wang and R. Ramirez-Mitchell, A Network of Transverse and Longitudinal Intermediate Filaments is Associated with Sarcomeres of Adult Vertebrate Skeletal Muscle. J. Cell Biol. 95 (1982) Part 2, 234 a.

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NEW EQUIPMENT

A Phillips 420 electron microscope with equipment for image processing is being installed in the laboratory of Dr. Malcolm Brown.

NEW FACULTY AND/OR STAFF

Dr. Tako Itoh of Kyoto, Japan is a visiting faculty member in the Department of Botany. Dr. Sharda Saradambal has a postdoctoral position in Dr. Malcolm Brown's laboratory.

OTHER NEWS OF INTEREST TO MEMBERS OF TSEM

Dr. W. Gordon Whaley, Ashbel Smith Professor of Cellular Biology, died the night of December 14, 1982, at the age of 68. Professor Whaley was a foremost authority on the Golgi Apparatus and one of the first to describe this organelle in plants. He was indeed a father of electron microscopy of plant systems in the United States, and greatly influenced development of electron microscopy in Texas. His tenure at UT included terms as Chairman of the Botany Department, Dean of the Graduate School and Director of the Cell Research Institute. Many of us in TSEM knew him as an excellent teacher, mentor, and friend. Prof. Whaley had planned to retire in 1984.

COLLEGE OF PHARMACY, PHARMACOLOGY

OTHER NEWS OF INTEREST TO MEMBERS OF TSEM

Dr. Daniel Acosta and Dr. Elsie Sorensen have received a grant from the Johns Hopkins Center for Alternatives to Animal Testing (director, Dr. Alan Goldberg), entitled Hepatotoxicity: An **In Vitro** Approach to the Study of Toxicity and Membrane Interactions of Cadmium Using Cultured Rat Hepatocytes.

Call Home Memo #1

To: All TSEMJ Readers
From: T.E.*

Message: Why don't you send us one of your manuscripts (Research Reports, Technical Notes, or Historical Reviews) for publication? We really need your input! Without them, T.E. really will have to "Go Home".

*The Editor (TSEMJ)

Announcements

(1) Texas Society for Electron Microscopy's Annual Spring Meeting — 1983

April 7-9, 1983
Quality Inn in Austin

Guest Speaker: John Guyton, Baylor College of Medicine

Agenda: Campus Tours
Social Events
Podium and Poster Sessions

Registration: $22/R; $12/S; $32/NM

Contact: C.P. Davis
Program Chairman, TSEM
Dept. Microbiology
U.TM.B.
Galveston, Texas 77550

(2) Texas Society for Electron Microscopy's Annual Fall Meeting — 1983

October 13-15, 1983
Sheraton Inn — Tyler, Texas

Guest Speaker: to be announced

Agenda: Rose Garden Tour
Social Event (s)
Poster and Platform Sessions

Local Arrangements: Ron Dodson

Contact: Ernest F. Couch
Program Chairman, TSEM
Department of Biology
Ft. Worth, Texas 76129

(3) Eleventh Western Regional Meeting of Electron Microscopists

May 11-14, 1983
Asilomar, California

Contact: Caroline Schooley
E.M.L.
1581 Life Science Bldg.
University of California
Berkeley, CA 94720

(4) EMSA/MAS Annual Meeting — 1983

August 6-12, 1983
Hyatt Regency and Adams Hilton
Phoenix, AZ

Local Arrangements: C. Ward Kischer
Department of Anatomy
College of Medicine
University of Arizona
Tucson, AZ 85724

Contact: Pat Calarco
Department of Anatomy
University of California
San Francisco, CA 94143

(5) American Society for Cell Biology's Annual Meeting — 1983

November 29-December 3, 1983
Convention Center-San Antonio, Texas

Local Arrangements: Bob Turner
Department of Pathology
Scott and White Clinic
Temple, Texas 76508
(817) 774-3688

Contact: Professional Associates
2012 Big Bend Boulevard
St. Louis, MO 63117

Scanning And Transmission Electron Microscopy

A series of practical courses will be offered during June 1983.

Transmission Electron Microscopy . . . . . June 6-17
Scanning Electron Microscopy . . . . . June 20-24
Combined TEM & SEM . . . . . June 6-24

These classes are designed to introduce the participants to the theory and practical aspects of electron microscopy. Primary emphasis will be on specimen preparation, operation of electron microscopes, X-ray microanalysis, and photographic and darkroom techniques.

Write or call:

Fred Lightfoot, George Washington University, Department of Anatomy, 2300 I Street, N.W., Washington, D.C. 20037, (202) 676-2881 or 676-3511

Dear TSEM Member:

In October the nominating committee of your society met to nominate two candidates for each office that will be filled by election this spring. We are pleased to announce the following slate of candidates:

President Elect:	Allen Shannon Leon McGraw
Program Chairman Elect:	Howard Arnott M. Lynn Davis
Treasurer:	Randy Moore Joiner Cartwright

Sincerely,

Elizabeth Root

TSEM By-laws

Article I — NAME

The name of the Society shall be the Texas Society for Electron Microscopy.

Article II — PURPOSE

This Society is organized exclusively as a scientific and educational organization. The purpose of this Society shall be (a) to increase and disseminate knowledge concerning the biological and physical applications of electron microscopy and related instrumentation and (b) to promote free exchange of ideas and information among electron microscopists and interested participants. Not withstanding any other provision of these articles, this society shall not, except to an insubstantial degree, engage in any activities or exercise any powers that are not in furtherance of the purposes of this society. No substantial part of the activities of the Society shall be the carrying on of propaganda, or otherwise attempting to influence legislation, and the Society shall not participate in, or intervene in (including the publishing or distribution of statements) any political campaign on behalf of any candidate for public office.

Article III — MEMBERSHIP

Membership in the Society shall be open to individuals who share the stated purpose of the Society. The Society shall consist of regular members, student members, corporate members, and honorary members.

An applicant, other than a corporate organization, having an interest in electron microscopy may be considered for regular membership. An applicant enrolled in an academic undergraduate or graduate program will be considered for student membership. Students wishing to become more involved in the Society may elect to apply for regular membership. Any applying commercial organization having an interest in electron microscopy shall be considered for corporate membership. A corporate membership shall entitle that corporation to designate one representative who shall receive membership benefits as a regular member. Other representatives of the same organization may apply for regular membership to receive Society privileges. Honorary membership shall be restricted to either (a) distinguished scientists who are not members of the Society, but who have made significant contributions to this Society or (b) to Society members for extended and outstanding service to this Society.

Application for regular, student, and corporate membership shall require nomination by any regular member in good standing and shall be made to the Secretary, who, with the approval of the Executive Council, shall report same at the next business meeting of the Society. A two-thirds vote of the regular members present shall elect applicants to membership.

Nominations for honorary membership may be made by any member of the Society. Nominations shall be made in writing to any member of the Executive Council and must be accompanied by written evidence of the nominee's eligibility. The member of the Executive Council shall present the nomination at the next meeting of the Executive Council for consideration. The Executive Council shall act upon the nomination within one year of its presentation and shall notify the nominator of the final action taken on the nomination.

Only regular members shall have the right to vote, to nominate new members, to hold office, or to serve on committees. Corporate members may exhibit at the Society's meetings (additional exhibition charges may be levied by the Executive Council). An honorary member shall be exempt from dues and shall be entitled to all privileges of regular membership. All members shall receive Society mailouts except for ballots which will be mailed only to regular members.

The amount of dues shall be set by the Executive Council. Dues shall become payable on January 1 of each year. Members unpaid by the Spring meeting shall be notified and if still unpaid will be dropped from membership after the Fall meeting.

Article IV — OFFICERS

A. Elected Officers

The elected officers of the Society shall be President, President-Elect. Immediate Past President, Secretary, Treasurer, Program Chairman, and Program Chairman-Elect. The President-Elect shall serve one year as such, one year as President, and one year as Immediate Past President. The Secretary shall be elected in even-numbered years and serve for a two year term. The Treasurer shall be elected in odd-numbered years and serve for a two year term. The Program Chairman-Elect shall serve one year as such, followed by one year as Program Chairman. The installation of incoming officers shall be at the Spring meeting. All officers shall arrange for the orderly and timely transition of their offices within 30 days after the installation of officers. However, all officers shall continue until relieved by their successors. The duties of the officers shall

1. **President:** shall preside at all business meetings of the Society and at meetings of the Executive Council. The President shall represent the Society at the annual meeting of the Electron Microscopy Society of America. The President shall conduct the business of the Society between Executive Council meetings.
2. **President-Elect:** shall assist the President and substitute for him in his absence and perform such duties as assigned by the President.
3. **Immediate Past President:** shall assist the President and Executive Council.
4. **Secretary:** shall maintain the records of the Society other than financial, and distribute announcements to the membership.
5. **Treasurer:** shall be custodian of the Society funds and shall account for them in accordance with accepted business practice. The Treasurer shall be bonded and the cost of such shall be borne by the Society. The Treasurer shall have his records examined annually by an internal audit committee chosen by the Executive Council at the Winter meeting. A written report of the internal audit shall be presented to the Executive Council and the membership at the Spring meeting.
6. **Program Chairman:** shall be responsible for organizing the various scientific activities of the Society. The Program Chairman shall not commit any funds of the Society unless authorized by the Executive Council via an approved budget or as authorized by the President and Treasurer under conditions of exigency.
7. **Program Chairman-Elect:** shall assist the Program Chairman and substitute for him in his absence and, additionally, extend the planning of programs into his own term of office as Program Chairman.

B. Appointed Officers

The appointed officers of the Society shall be the Newsletter Editor and the Student Representative who shall be appointed by the Executive Council.

1. **Newsletter Editor:** shall publish a Newsletter three times a year promoting the purpose of the Society, unless otherwise ordered by the Executive Council. The term of appointment shall be for two years and may be renewed.
2. **Student Representative:** shall represent the student membership of the Society on the Executive Council. The term of appointment shall be for one year.

Additionally, the officers of the Society shall perform the duties prescribed by the Bylaws and, as appropriate, by the parliamentary authority adopted by the Society. No part of the net earnings of the Society shall inure to the benefit of, or be distributable to its members, trustees, officers, or other private persons, except that the Society shall be authorized and empowered to pay reasonable compensation for services rendered and to make payments and distributions in furtherance of the purposes set forth in Article Two hereof.

Article V — MEETINGS

There shall be three scientific meetings per year: fall, winter, and spring, unless otherwise ordered by the Society or by the Executive Council. Exact times and places of these meetings shall be designated by the Executive Council. A business meeting will be held at each scientific meeting of the Society. Parliamentary procedures to be followed in the business meeting shall be those specified in the current edition of **Robert's Rules of Order Newly Revised.** Ten percent of the regular

members, or 35 regular members, whichever is smaller, shall constitute a quorum at a business meeting.

Article VI — EXECUTIVE COUNCIL

The Executive Council shall be responsible for the scientific and administrative obligations of the Society. It shall determine policies for the good of the Society in accordance with these By-laws; it shall plan scientific and business meetings, it shall authorize the expenditure of Society funds, and it shall conduct other duties as required for the benefit of the Society. The Executive Council shall meet prior to the business meeting at each scientific meeting of the Society. Special meetings of the Executive Council can be called by the President and shall be called upon the written request of three elected members of the Executive Council.

At each spring meeting the Executive Council shall appoint a Student Representative who shall represent the student membership of the Society on the Executive Council the following year as a voting member. The Executive Council shall also appoint Local Arrangements Chairmen for each of various meetings and in so doing shall duly consider the recommendations of the PROGRAM Chairman and the President. Local Arrangements Chairmen are **ad-hoc**, non-voting members of the Executive Council.

Executive Council meetings are open to the membership.

The elected and appointed officers shall constitute the Executive Council. The President and four other elected officers or the President-Elect and four other elected officers shall constitute a quorum.

Article VII — COMMITTEES

Standing or special committees shall be appointed by the President as directed by these By-laws or as the Society, or the Executive Council, shall from time to time deem necessary to carry on the work of the Society. The President may appoint advisory committees at any time without prior consultation with the Executive Council. The President shall be **ex officio** a member of all committees except the Nominating Committee.

Article VIII — ELECTIONS AND INTERIM VACANCIES

In February of each year the Executive Council shall appoint three regular members to serve on the Nominating Committee with the President-Elect, and the Secretary. The Secretary shall serve as chairman of the Nominating Committee. The Nominating Committee shall nominate two candidates for each officer position becoming vacant that year. In preparing the slate of nominees, due consideration shall be given to the geographical area and fields of interest represented by the membership of the Society and to the nominees previous participation in the Society's affairs. The Nominating Committee shall also ascertain the willingness of each nominee to serve if elected. The report of the Nominating Committee shall be announced to the regular membership by March 1.

Additional nominations may be initiated by the membership by a petition to the Secretary signed by a minimum of ten of the regular members. Such petitions must be received by the Secretary by March 15.

Ballots shall be mailed to the regular members in March and completed ballots shall be accepted by the Secretary until April 15. The Secretary shall count the ballots on the next appropriate day and announce the results of the election at the spring business meeting and by mailout to the regular membership. Any regular member may examine the ballots at the spring business meeting.

The candidate receiving the largest number of votes shall be the winner. In the event of a tie vote, the Executive Council shall decide the winner. The ballots shall be examined by the Executive Council at the spring meeting.

A two-thirds vote of the entire membership of the Executive Council shall remove any officer or appointee derelict in their duties. The Executive Council shall accept resignations in good faith.

An interim vacancy in the presidency shall be filled by advancement of the President-Elect, who will go on to serve his anticipated term as President and Immediate Past President. In the event there is no President-Elect to advance, the Executive Council shall elect one of its members as acting President to serve until the completion of the next regular election. An interim vacancy in the office of Program Chairman shall be filled by the Program Chairman-Elect, who will go on to serve his anticipated term as Program Chairman. If there is no Program Chairman-Elect to advance, the Executive Council shall appoint a Program Chairman to serve until the completion of the next regular election. Interim vacancies in the offices of Secretary or Treasurer shall be filled by appointment by the Executive Council until the completion of the next regular election. Interim vacancies in the offices of Newsletter Editor or Student Representative shall be filled by an appointment made by the Executive Council.

Article IX — DISSOLUTION

Upon the dissolution of the Society, the Executive Council shall, after paying or making provision for the payment of all the liabilities of the Society, dispose of all of the assets of the Society exclusively for the purposes of the Society in such manner, or to the Electron Microscopy Society of America. Any such assets not so disposed of shall be disposed of by the Court of Common Pleas of the county in which the principal office of the Society is then located, exclusively for such purposes or to such organization or organizations, as said Court shall determine, which are organized and operated exclusively for such purposes.

Article X — AMENDMENTS

Amendments to these By-laws may be initiated by individual members of the Executive Council or by petition to the Secretary signed by ten regular members of the Society. Amendments must be approved by a two-thirds majority of the Executive Council, the proposed amendment shall then be promptly submitted by mail to the regular membership by the Secretary with statements of support and/or opposition by the Executive Council. The ballots shall be accepted by the Executive Council for one month after the date of mailing. The Executive Council shall count the ballots; the amendment(s) shall be ratified if it receives a favorable two-thirds majority of the votes cast. Any regular member can, if he so desires, be present at the counting of the ballots.

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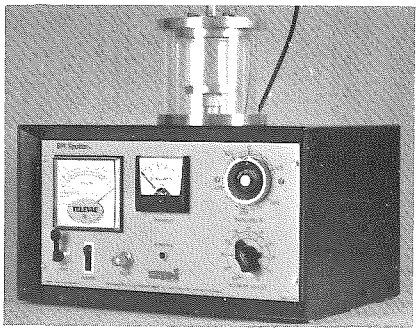

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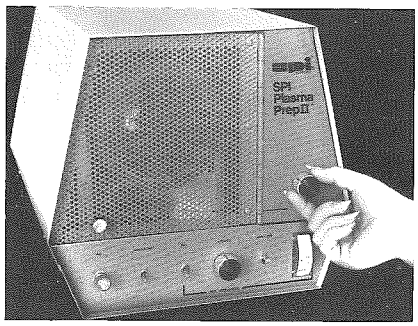

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