University of Nebraska Medical Center
BREAKTHROUGHS FOR LIFE

University of Nebraska Medical Center
DigitalCommons@UNMC

MD Theses

Special Collections

1961

Principles involved in electron microscopy

Daniel Keith Roberts
University of Nebraska Medical Center

This manuscript is historical in nature and may not reflect current medical research and practice. Search PubMed for current research.

Follow this and additional works at: https://digitalcommons.unmc.edu/mdtheses

Recommended Citation

Roberts, Daniel Keith, "Principles involved in electron microscopy" (1961). *MD Theses*. 2571.
https://digitalcommons.unmc.edu/mdtheses/2571

This Thesis is brought to you for free and open access by the Special Collections at DigitalCommons@UNMC. It has been accepted for inclusion in MD Theses by an authorized administrator of DigitalCommons@UNMC. For more information, please contact digitalcommons@unmc.edu.

PRINCIPLES INVOLVED IN ELECTRON MICROSCOPY

Daniel Keith Roberts

Submitted in Partial Fulfillment for the Degree of
Doctor of Medicine

College of Medicine, University of Nebraska

April 1, 1961

Omaha, Nebraska

PURPOSE

Since the time of the discovery of the light microscope, scientists have continually attempted to better their methods of viewing minute structure. One of the most remarkable advances is the electron microscope. With the entrance of the electron microscope into the field of biological science came many new problems. Histologists found themselves in a position in which they were forced to conceive an entirely new perspective in the relationships of one ultramicroscopic structure to another, and to correlate this with their present knowledge of histology. Likewise, with all of this came an entirely new philosophy, new attitude, and a new horizon to explore. In this paper I shall attempt to portray in a methodical manner the most recent, properly evaluated methods and techniques involved in learning the art and science of electron microscopy. It is intended to be of service to those interested in learning electron microscopy for use as a scientific method of investigation.

494741

ACKNOWLEDGMENTS

Throughout his many years in the Department of Anatomy at the University of Nebraska College of Medicine, Dr. J. S. Latta has stimulated the growth of many embryo physicians to their place in the Art and Science of Medicine. I feel indeed fortunate to have had the opportunity of close association and the benefit of expert guidance and knowledge in the culmination of the research, review, and construction set forth in this paper.

I would also like to acknowledge the many helpful suggestions of Dr. R. B. Wilson, Department of Pathology and Dr. E. A. Holyoke, Department of Anatomy; and, for his technical assistance and suggestions Mr. David Rhea, Department of Microbiology. I am indebted to all members of the Department of Anatomy for their fine cooperation and assistance during this project.

TABLE OF CONTENTS

		Page
I.	Introduction	1
II.	Prefixation Specimen Preparation	6
	A. Special Problems	6
	B. Techniques	8
	C. Figure 1	
III.	Fixation	16
	A. Osmium Tetroxide	16
	B. Potassium Dichromate	24
	C. Potassium Permanganate	25
	D. Others	26
	E. Qualities of Good Fixation	27
	F. Figure 2	
IV.	Embedding	29
	A. Dehydration	29
	B. Methacrylate	30
	C. Epoxy Resins (Araldite & Epon)	33
	D. Polyesters (Vestopal W & Selectron)	36
	E. Prepolymerization & Polymerization Damage	38
	F. Figure 3	
V.	Sectioning	40
	A. Microtomes	40
	B. Trimming & Mounting Specimen Capsule	41

C. Preparation, Selection & Mounting of Glass Knives 42

D. Diamond Knives. 45

E. Sectioning "per se" 46

F. Thickness & Quality of Sections 47

G. Common Errors in Sectioning 52

H. Figures 4-10

VI. Staining-Specimen Carriers-Specimen Mounting..54

A. Staining 54

B. Specimen Carriers (Holders or Grids) . . .56

C. Specimen Mounting. 59

D. Figures 11-12

VII. The Electron Microscope 62

VIII. Practical Theory of the Electron Microscope . 65

A. Electro-Magnetic Lenses 65

B. Electro-Magnetic Lenses Correlated into the Vacuum System of an Electron Microscope 66

C. Micrographs 68

D. Vacuum Pumps. 68

E. Figures 13-14

IX. Operation of the Philips Electron Microscope-100B 70

A. Instruction 70

B. Figures 15-17

X. An Application to Research 79

A. Introductory Remarks 79

B. Historical Review 79

C. Techniques & Methods. 80

D. Observations & Discussion 80

E. Conclusions 81

F. Figures 18-25

XI. Summary & Conclusion. 83

XII. Bibliography

I.

INTRODUCTION

Electron microscopy should be used as a tool of scientific research in correlation with the acquired and innate knowledge of the individual investigator to obtain insight into the structure of living matter. The author believes that it can be most properly applied for measurement of the end result of investigations, not only in this field of Anatomy and Pathology, but also in other fields such as Endocrinology, Biochemistry, Bacteriology, Physiology, Pharmacology and Clinical Medicine. The contributions made in the field of research today are made primarily by probing the unknown and by using a methodical basic science research approach. I believe this is adequately illustrated in the field of Cancer research where, in the beginning, most research work was done on the higher clinical levels. Now we have been forced to return to the basic science level to follow through, in a methodical step by step approach, from the basic problem, i.e., the alteration of morphology either as regards its histological or pathological growth, its metabolic phenomena or its physiological development. It is like the construction of a good house. One cannot start on the second floor, but rather must begin at the basement level. Therefore, in adopting a proper attitude toward electron microscopy one must realize what the end result will be on the top floor of the building he is constructing, rather than the day to day accomplishments he attains.

As is true in many fields of research, many administrators and clinicians are apt to have some inkling of the "wonders" of electron microscopy, but little knowledge of its problems. They think of electron microscopy as a simple extension of light microscopy, and assume that problems that can be straightforwardly dealt with in the latter instance should be all the better answered by the new technique. One has to convince these people that problems in electron microscopy inevitably proceed at a much slower pace so that the "simple" problem which might take only weeks with a light microscope may take years with the electron microscope. It must be emphasized that the sluggishness of the project has nothing to do with one's energies or abilities, but is intrinsic to the method. All people in an institution where an electron microscope has recently been installed must be educated to its intrinsic faults and incapabilities. In such a manner the author hopes to inform those reading this manual so that they might in the future help to educate others around them.

The following dimensional figures may help explain the problem. If a standard "200" mesh supporting grid is used, there is about one square millimeter of open area which can be viewed by the electron microscope. Assuming that this square millimeter is covered by perfect sections, which would be quite unusual, it would take several hundreds of these grids to be equivalent to the ordinary tissue section which the pathologist may deal with in five minutes or less. When one has excellent material, it is not unreasonable

for him to spend four to eight hours or more on a single grid. During this period one square millimeter, or less, of tissue surface is viewed and one cannot possibly photograph more than a tenth of this, even at low magnification. Judging from this, it would take a solid year of work to record what the light microscopist can dismiss in a brief period.

To illustrate this in another way, it we take a picture at 1500X on a two inch plate, approximately 0.001 mm^2 of tissue surface area is recorded. Seldom does one take more than fifty pictures in a single day. Even at this low average magnification, it would take almost a month at the microscope to photograph one square millimeter, and seven and one-half years of continuous photography to photograph one square centimeter.

Considering the third dimension, the problem, of course, becomes much worse. Useful sections can be no greater than 0.5 micron thick. At this thickness, twenty serial sections traverse a single micron, possibly spanning the thickness of one mitochondrion. Approximately one hundred serial sections would be required to span a nucleus, and about two hundred serial sections to cross the thickness of a single small cell. In conventional light microscopy an embryologist seldom needs as many sections to go through an entire pig embryo!

"An electron microscopist who is concerned with tissue work simply cannot accept most problems that are primarily statistical. He cannot possibly process enough samples. He can only effectively deal with problems which yield simple qualitative answers. He must

design experiments so that a relatively few samples will be significant. It is not only the administrator and clinician who sometimes must be educated in this, but also the ambitious beginner in electron microscopy who must learn to limit his objectives and define them carefully.

"Electron microscopy received widespread publicity in its early stages and undoubtedly many people were naively led to expect too much. It has its limitations! In essence one must pay for magnification by accepting a restriction of field and all that this implies. One cannot do conventional histology with an electron microscope any more than one can do gross anatomy with a light microscope. The world of the electron microscope is a world that could only be studied inferentially before. It is a vastly important world and we should hardly have to defend its exploration with the most effective tool to date. The flea scurries about and his whole world is less than one square yard of a dog. The electron microscopist can be very busy too, and in his whole lifetime not cover as much territory." (Pease, 1960)

It is inherent, then that the student in electron microscopy recognize the scope of the task ahead of him. The difficulties of a novice getting started in this field result in large measure from his overlooking seemingly small, but usually critical detail. Often, also, it is a result from the necessity of performing all sorts of the essential activities on a much finer scale than most people have had practice with. A jewelers work is coarse by comparison.

The unaided eye is quite inadequate. Binocular dissecting microscopes and binocular headloops are often necessary to allow the electron microscopist to properly carry out his work. It will be a difficult adjustment for a person who has been dissecting with stiff blunt forceps and who has been trimming paraffin blocks with a dull scalpel to refine his tools as well as his movements to such a fine state. In this respect the person who has done no conventional microscopy may have less a problem. I believe the most important thing for the student to remember is that the attention paid to the fine intricacies of detail will eventually measure the resultant skill, adaptability, and art of the formed electron microscopist.

I believe that through the preceding introduction it is easily recognizable that investigation with the electron microscope on a project basis by an individual is not best carried out on a shared facility basis. While the practicality of only one or two investigators in the same field using the same electron microscope is not readily seen by most administrative officials, the ratio of the resultant output to the financial investment would be much greater over a significant period of time if this were the case. It is hoped by this author that eventually this will be a fully recognized fact and more electron microscopes will be available for those trained to use them.

II.

PREFIXATION SPECIMEN PREPARATION

Multiple problems meet the student in this first step of preparing tissues. He must fully realize that the resultant of his efforts in preparing a specimen will be only as good as his weakest link. Since a lot of time is involved in the complete preparation of a specimen, he should be meticulously careful each step of the way. A second, much more important motivating factor, is that each specimen may, in its minute structure, contain a significant finding that is not present in any other specimen. Even if one finds this in a poorly prepared specimen at the end of the process, it may be significantly distorted, or, it may not be possible to differentiate from artifact. Too many times this author has carried out a complete preparation of very important material only to find out that somewhere in the process he had destroyed an important membrane or intracytoplasmic structure. Thus the author reemphasizes, from the killing of the animal to the viewing of the electron micrographs, be meticulous in explicitly following the details.

The biggest problems I encountered in this first step were: 1. Adequate exposure to osmium tetroxide in a significantly short period of time. 2. Mechanical destruction due to improper mincing or slicing. 3. Chemical destruction due to the use of saline or other fluids prior to fixation. 4. Inadequate respect of the deactivating action of blood and tissue fluids. 5. Frequent corneal

fixation secondary to inadequate respect of the detrimental actions of osmium tetroxide fumes.

To further define these problems, the time interval from the dearterialization of the tissue at the beginning of fixation should take no longer than one minute and preferably no greater than thirty seconds. Any further delay than this allows disruption of structures vital to the electron microscopist.

As regards mechanical destruction, it is not always due to improper technique, but, by very meticulous technique, this hazard can be brought to a minimum. This technique will be discussed and the problem itself is relatively self explanatory. It is mostly brought about by the shearing force of the razor blade on the tissue.

Chemical destruction is that destruction brought about by the use of "flushing" fluids of improper isotonic and isosmotic values. The fixative also falls into this category. It affects primarily the intracytoplasmic structures with leaching and, in particular, swelling and shrinking of mitochondria.

The deactivating action on osmium tetroxide by blood and tissue fluids is now well known. This must be considered whenever there is a large amount of these fluids present in tissue and is particularly troublesome in the vascular perfusion method. It should be mentioned here that metals tend to precipitate osmium tetroxide so that when using razor blades for cutting or other metal instruments for handling tissues in the early stages, this becomes a factor.

The dangerous aspects of osmium tetroxide should be strongly

emphasized. This is a dangerous chemical which may affect each individual differently. Its most noted site of attack is the corneal conjunctival epithelium. The fumes of this chemical readily fix epithelium as they would any other tissue. Symptomatology of this fixation is: 1. A progressive burning of the eyes, and 2. The appearance of a haze or fog covering all that one sees three or four hours after exposure. Contrary to what other authors have said, this can occur with very minute exposure, the amount of which varies with each individual. One is not necessarily safe if during the procedure he does not smell the fumes of the osmium, for this is not a prerequisite for corneal fixation. Some people have idiosyncratic reactions to this substance such as rhinitis, excessive lacrimation and periorbital swelling. The chemical will cause fixation of skin if one spills it or gets close exposure to fumes. This causes a brownish-black, probably nonharmful discoloration. Before leaving this subject, I would again repeat that this is a dangerous chemical and should be used cautiously.

Numerous methods of prefixation preparation are available. The oldest was brought to my attention in Palade's article in 1952. One removes the tissue from the anesthetized animal in a long narrow strip (5-10 mm. x 1 mm.) and places it on a piece of cardboard or filter paper. One immediately places several drops of osmium tetroxide over the tissue, starting the fixation process. One then quickly slices this tissue with a razor blade into one millimeter sections yielding pieces of tissue no larger than one cubic millimeter and

preferably smaller. These then are immediately placed in the fixative solution for completion of the fixation process.

This method has several shortcomings, but is applicable with some modification. First, while cutting this into small pieces, one must protect himself against the osmium tetroxide fumes by wearing airtight goggles or by working with the tissue under a hood. Likewise one should protect himself from discoloration of his skin by wearing gloves, either rubber or plastic.

A second problem is the proper exposure of the tissue surface to active osmium tetroxide fumes. This problem may be surmounted by using a paraffin-molded shallow container instead of cardboard. In this, one can have much more fixative covering this specimen and can continually flush this specimen while cutting, so that the available amount of active osmium tetroxide is greater.

The problem of tissue fluid deactivation or dilution is decreased. This will somewhat prolong the time available for slicing the strip in a more precise, accurate manner. This does not, however, solve the problem of precipitation and the time available at this point is still somewhat limited.

If one attempts to decrease the fluid factor by flushing the tissue with a salt solution, he may run into further intracytoplasmic structural distortion. Substances used for this must be isotonic and must have the same ionic dissociation as tissue fluids. Without this, one may get despiralization of chromosomes, chromatid and spindle disorganization along with chromosomal and mitochondrial

swelling. Most of this is thought to be due to low osmotic pressure fluids, but when isotonic solutions are used, the chromosome swelling found was thought by Hungerford and DiBerardino (1958) to possibly be due to delay of fixation.

The mechanical disruption one gets with this method is relatively high, particularly in some tissues such as nervous system tissue. It can somewhat be decreased by the time taken to cut the tissue carefully. One should use new razor blades cleaned in acetone for each new piece of tissue.

This method is poorly applicable to some tissues such as spleen and brain, even with modifications. With liver and many other tissues, however, this works relatively well and is widely used.

As already stated, the above described is the old standard method still used by many for most tissues. In our laboratory and others, three other methods have been attempted with fair results in some, and good results in others, depending on the tissue and the method.

The three following methods are "in vivo" methods so that fixation can take place without loss of vital blood supply. The first is that of fixing a surface of an organ in an anesthetized animal prior to removal. This is accomplished by stripping away the surface capsule which leaves a freely oozing surface. One then flushes this with several milliliters of properly prepared fixative solution until the oozing stops. This begins immediate fixation without deactivation or dilution by the tissue fluids. One then

places a small piece of cotton over this area and soaks this with the fixative solution. This allows a continued bath of solution over the area of desired fixation. Two to three drops of fixative are added to this cotton every two to three minutes for about fifteen minutes. Then the area can be cut in a thin slice from the organ, diced into one millimeter cubes and placed in the fixative solution for the rest of the desired fixation time. The separation of this area is relatively easy because the fixation has caused hardening of the penetrated area. This method is accompanied by the same hazards as the others as regards danger of osmium tetroxide fumes. Proper precautions as before listed are necessary. The tissue fluid deactivation property has been coped with by constant flushing with the fixative. The mechanical disruption problems are less here because this fixation occurs prior to cutting. The chemical disruption is unlikely if the fixative is properly prepared. Most important of all, the time interval from dearterialization to fixation is almost zero seconds. It is obvious, of course, that all organs cannot be dealt with in this manner for many receive their blood supply through the capsule. The brain, kidney, and with proper handling, the spleen may be fixed by this method. The adrenal cannot be.

Another method is that of creating a free surface for fixation. This is particularly useful when deeper structures are desired. This method and a modification thereof, is the best method applicable as far as the author is concerned, to spleen, kidney, and liver. This is actually only a modification of the above, but here a wedge of

tissue is removed from the surface of an organ to the desired depth. Then the fixative is flushed through the area until the oozing stops; again, cotton is applied and the area is kept bathed in frequently replenished osmium tetroxide fixative solution. One may even cut completely across an organ with this method, but bleeding becomes a particular problem in this case. The only disadvantage of this procedure over the previous one is the possibility of mechanical disruption along the original cutting edge and the fact that more vigorous flushing with fixative is required.

A modification of the above method has been tried and proven useful in this laboratory. The idea for this method was submitted by a graduate student associate, Jerome H. Smith, and was tested by myself and Mr. Smith in the experimentation done in preparation for my Master's thesis. One takes six to eight razor blades, single edged, and first cleans them in an acetone solution and wipes them dry with a lint-free cloth. They are then placed side by side with the sharp edges oriented in this same direction. On each handle side of the razor blade one or more strips of masking tape are placed as spacers so that the edges are parallel to one another and approximately 0.5 to 1.0 millimeter apart. The entire handle portion of these razor blades are then taped sturdily together with masking tape and the resultant is a tandem razor blade with six to eight razor sharp edges parallel to one another, on the same plane, approximately 0.5 to 1.0 millimeter apart. (Figure 1) One then exposes

the organ to be cut, draws the blade quickly across the organ in a circular motion consisting of a short down stroke and quick but longer upstroke. This leaves six to eight cuts about one millimeter apart and approximately two millimeters deep into which the fixative is quickly flushed in copious quantities. Following the stoppage of oozing, the fixative is continuously applied to the incisions by placing a small amount of cotton over the entire portion and keeping it supersaturated with fixative solution. At the end of fifteen minutes the several cut strips are removed in block. Separation of fixed from unfixed tissue is easy because of the hardening. The tissue is then cut across at 0.5 to 1.0 millimeter intervals to again prepare the one cubic millimeter tissue blocks. This may be done with a single blade or with the previously described tandem razor blade. Another way of using this instrument, which is of equal effectiveness, is to make the "in vivo" cuts, remove the strips immediately, separate the strips and place them in a shallow, paraffin container bathing the tissue with fixative; and, then, after fifteen minutes of fixation, cut them into one millimeter cubes and place them in another solution for completion of the fixation process. This latter method appears to cause greater mechanical destruction, however. May I reemphasize that all of this should be done under a hood, or one should wear airtight goggles. Both of the methods using the tandem razor blade have proven very effective in our laboratory. Skill in using this instrument takes the sacrifice of about one animal with practice on the various organs in that

animal. The only real drawback of this method is the requisite use of a somewhat larger amount of fixative.

To facilitate the above procedures so that a minimum amount of postmortem changes take place prior to fixation, and to keep the tissue at an optimum temperature for fixation, the organ should be identified, isolated, and mobilized into such a position that it can be packed in ice during the procedure. A minimal amount of trauma during this mobilization is essential. This is especially true in organs such as the spleen which are dynamic in their reaction to trauma. If not, one will be viewing pathologically or functionally altered morphology which is iatrogenic.

There are other methods used in various special instances. Vascular perfusion, for instance, has found some use in such tissues as brain, liver, and possibly spleen. An almost insurmountable problem here, however, has been the deactivation of osmium tetroxide by the blood plasma. It requires very large amounts of fixative solution and even then may be only partly successful. Arterial injection in this perfusion is not the route of choice because osmium tetroxide is a marked vasoconstrictor and the fixative gets no further than the constricted arterioles. Pease (1960) has found vasodilators of no assistance. Injection needs to be retrograde via a venous vessel with a continuous drainage route supplied. The author has, as yet, no successful experience with this in its application towards splenic fixation, but excellent micrographs of liver

cells by vascular perfusion have been published. Cerebral spinal fluid channels have also been utilized in this way.

Pallie and Pease (1958) introduced the use of hyaluronidase as a pretreatment for penetration of tissue where connective tissue is thought to represent a formidable barrier. The author has had no experience with this and refers the reader to the original article. The authors found this method most helpful in the fixation of arteries and in the fixation of sweat glands in a rat's foot pad. The method is not as yet widely used but sounds promising.

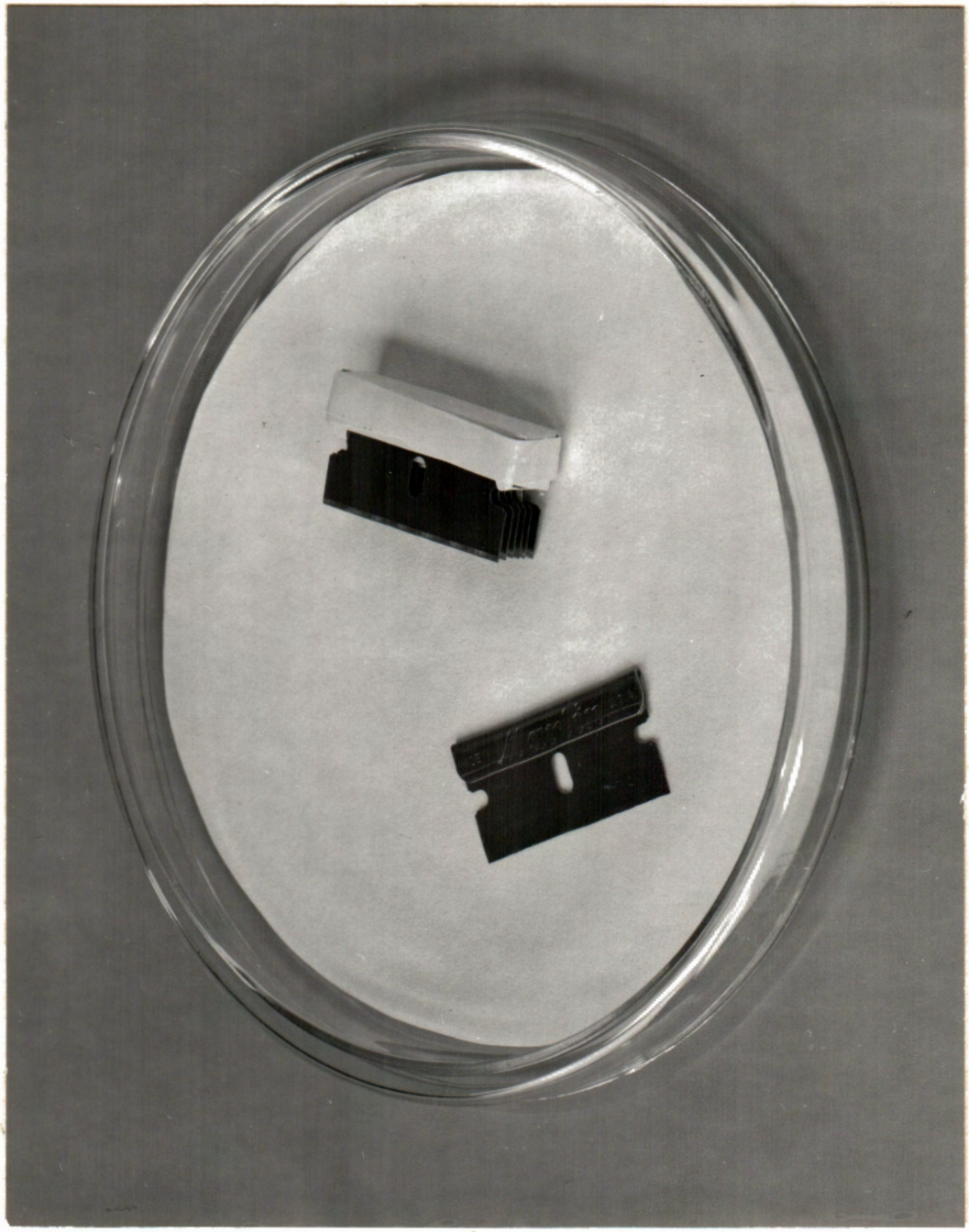
For further ideas regarding special problems concerning prefixation treatment, the student of electron microscopy must search his own imaginary mind as well as the literature which is rapidly increasing in volume each day.

Plate .

Chapter II.

Plate I.

Figure 1. Tandem Razor Blade (Smith, 1960).



III.

FIXATION

Osmium tetroxide is now considered to be the basic general all purpose fixative. This fixative was first used successfully in electron microscopy by Claude and Fullman in 1946. Pease and Baker in 1948 were next to successfully apply this fixative to ultrathin sections in preparation for the electron microscope. In 1950 the laboratories headed by Dalton, et al., Porter, and Pease and Baker did comparative studies of several common fixatives and this resulted in the unanimous opinion that osmium tetroxide was the general fixative of choice.

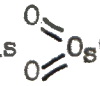
In 1952, after experimentation with various fixatives used by his contemporaries, G. E. Palade reported his formula for proper fixation. He studied extensively various influences of changes in the contents of the completed fixative solution. It is of utmost importance to be fully aware of why one is using such a precisely made solution. One should understand the reason for modification of old and new formulas rather than follow them "cookbook style". In this manner he will later have more insight into adaptations and adjustments he himself must make.

Palade considered the action of hydrogen ion concentration on the fixation process. He found that in tissue blocks osmium tetroxide fixation is preceded by an acidification wave. He felt this to be responsible for morphological alterations found at electron

microscopy. The etiology of this acidification he hypothesized to be two-fold. First, the production of acid by tissue enzymes secondary to trauma, or second the high hydrogen ion concentration of the osmium tetroxide fixative itself. He ruled out the first possibility by adding inhibitors of known acid producing metabolic processes such as anaerobic glycolysis and hydrolysis of phosphoric acid esters. This did not improve the result.

He next ran a series of experiments at various pH's, both buffered and unbuffered. The defects that he found were as follows: 1. Coarse precipitation of nuclear content at pH's less than 7.0. Nuclear content was more or less homogenous at pH of 7.0 to 8.0. 2. Mitochondria were swollen on both sides of neutrality. 3. Lipid inclusions were swollen and greatly vacuolated at pH's greater than 7.4. 4. Ground substance was coarsely granular at pH 5, finely granular with vacuoles at pH 6, homogenous with no vacuoles at pH7, and greatly vacuolated at pH 8. 5. Cell membranes, as well as the nuclear and mitochondrial membranes, appeared thicker and denser at acid pH's than at neutral or alkaline pH. He felt he showed conclusively that the appearance of cells fixed in tissue blocks by osmium tetroxide was highly dependent on the hydrogen ion activity of the fixing solution. He went on to show that the quality of fixation could be materially improved by buffering the osmium tetroxide solutions at pH 7.3 to 7.5 with the acetate-veronal buffer, which he considered the most applicable of the buffering systems. He recommended 1% osmium tetroxide buffered at pH 7.3 to 7.5 with

acetate-veronal buffer as the appropriate fixative for general purpose electron microscopy. This is known as "Palade's Pickle". Even in this early article it is significant that Palade suggests variation in molarity of substances as a possible cause of poor fixation.

To further understand osmium tetroxide as a fixative, I would like to refer to two other articles: Porter and Kallman (1953) and Bahr (1954). Porter and Kallman in their article state that the structural formula of osmium tetroxide is  . It is known to be neutral to litmus and is a non-electrolyte. For this reason the practice of calling it "osmic acid" is misleading and incorrect. Its action on various tissue differs in its rate and nature. The reaction with lipids is probably by way of an affinity of osmium tetroxide for the ethylenic double bond configurations, thus forming unstable osmium esters which decompose to deposit osmium oxides or hydroxides at the site of activity.

Reaction of Osmium Tetroxide with Ethylenic Double Bonds

Figure 2

They felt that little was known of the reaction of osmium tetroxide with proteins, but in their preliminary studies it was shown to produce, quickly, an initial gelation of protein solutions. They postulated that by virtue of its tetrahydral configuration, osmium

tetroxide may form polymer-like structures with proteins establishing linkages at double bond containing groups. This initial gelation may then be followed by a further oxidation and the production of soluble end products, which, after long fixation, wash out of the cell.

Bahr (1954) in his discussion of the action of osmium tetroxide suggested that the chemical action gave some indication of the affinity of this substance for different cellular structures. He readily admits that chemical cellular affinity is not necessarily a proven fact. He listed the following approximate order of reactivity as regards the chemistry of osmium tetroxide: "Sulfhydryl groups have a direct reducing action, which may partly account for reactions with e.g. peptides, proteins and enzymes. Double bonds show about the same reactivity, an outstanding property of osmium tetroxide. Reactions with olefinic chains are exothermic and fast, but the esters formed are rather liable to hydrolize into a diol and $O_sO_4 \cdot 2H_2O$. In ester formation with cyclic compounds, the osmium tetroxide is firmly bound to certain double bond positions in the molecule concerned and gives rather stable products. This reaction type is of interest for fats, waxes, lecithin, the cerebrosides, vitamins, certain hormones, gallic acids and all other biological substances having a basic sterol structure. With tertiary bases the osmium tetroxide molecule easily forms stable coordination compounds, which in turn are able to accelerate the double bond reactions

appreciably. Such coordination probably occurs with e.g. tryptophane. Amino groups in a terminal position and not salt linked, sulfide sulfur, hydroxyl and aldehyde groups in terminal positions and on certain carbon chain links, certain heterocyclic compounds and aromatic compounds with hydroxyl groups in juxtaposition are able to give intense reactions with osmium tetroxide, the latter being of importance when plant material containing tanning substances is studied."

Criegee, and the workers using his method, showed that a large number of the osmium tetroxide reactions with organic substances are affected by steric conditions. This fact explains in some cases the inertness of one and the reactivity of another compound both having very similar structures.

Bahr feels that the inertness of the carbohydrates and the nucleic acids towards osmium tetroxide is one of the most striking results obtained and may be an explanation of the nonimpregnated areas in cytoplasm and nucleus in ultrathin sections of osmium fixed tissues.

From empirical considerations it is suggested that protein reactions with osmium tetroxide are roughly dependent on the typtophane, cysteine and histidine /2 content in the protein. Some enzymes obviously form exceptions to the rule.

Even though a rough impression of the sites of osmium tetroxide and thus a vague interpretation of the osmium deposits in sections can be given, no conclusions can be drawn from the localization of

the osmium reactions in the heterogeneous cellular structures. The reactions of osmium tetroxide with biological material are dependent on time, temperature and concentration, and thus the results in the literature and Bahr's article suggest that the constancy of time, temperature, object size, and composition of the fluid are essential to get comparable results when osmium tetroxide is used for fixation.

Much remains to be done in understanding what is and is not fixed by osmium tetroxide but the above paragraphs give the reader some idea of the action of this chemical.

Palade's fixative formula, previously discussed, is as follows:

Stock Buffer Solution

Sodium veronal (barbital)	14.7 gm.
Sodium acetate	9.7 gm.
Distilled Water to make	500 ml.

Stock Osmium Tetroxide Solution 2%

Stock 0.1 Normal HCl ---------- 8.6 ml. concentrated HCl (36%, 11.6 molar) in water up to make one liter.

Fixative Solution

Buffer	5.0 ml.
0.1 Normal HCl	5.0 ml.
2% Osmium Tetroxide	12.5 ml.
Distilled water	2.5 ml.
(pH approximately 7.4)	

(Palade 1952)

Concerning his formula two important thoughts are of practical value: 1. Osmium tetroxide is very slow to dissolve and is best made up about twelve hours prior to use. It will keep for a couple of weeks in the refrigerator but I prefer to use up the solution in

one week or less. 2. The buffer solution keeps well but must be stored in the refrigerator. If it is not, molds and other micro-organisms will grow in the buffer.

This formula and its modifications is the most universally used fixative for electron microscopy. Essentially all students could start with this first original formula and not go far wrong.

Many workers in the field have modified this formula in various ways to produce a desired result. It was mentioned earlier that Palade in his early experimentation had noted that molarity may have some significance as regards the fixation. Caulfield (1957) felt that the hypotonicity of Palade's solution was of significance. He felt that sucrose when added to the standard Palade fixative at the rate of 0.45 gm. per ml. of a 1% buffered fixative solution, increased the tonicity without increasing the sodium ions and frequently resulted in better preservation of larger portions of the block than without the addition. In view of the findings of Hungerford and DiBerardino in 1958 on the effects of tonicity on cells, this seems to be a very necessary addition.

Palade in 1956 emphasized that certain ions should be avoided such as the ammonium ion, for it destroys the membranes of the endoplasmic reticulum. On the other hand, calcium and/or magnesium ions in little more than trace quantities may be advantageous in preventing the extraction of cement substances.

At the Karolinska Institute investigators have not felt that hydrogen ion strength was most important and, instead, emphasized tonicity. Zetterquist in 1956 gave us this modified formula:

Stock Buffer Solution
(Same as Palade's)

Stock Salt Solution

Sodium Chloride	40 gm.
Potassium Chloride	2 gm.
Calcium Chloride	1 gm.
Distilled Water to make	500 ml.

Fixative Solution

Buffer Solution	10.0 ml.
Salt Solution	3.4 ml.
0.1 Normal HCl	11.0 ml.
Distilled Water to make	50.0 ml.
Osmium tetroxide (dry)	0.5 gm.

This fixative should have a pH of 7.2 - 7.4 and its tonicity is estimated to be 0.34 molar.

In our laboratory we have emphasized the tonicity as being very important and have used a combination of Zetterquist's and Caulfield's formulae with the following formulation:

Stock Buffer Solution
(Same as Palade's)

Stock Salt Solution
(Same as Zetterquist's)

Fixative Solution

Buffer Solution	10.0 ml.
Salt Solution	3.4 ml.
* 0.1N HCl	6.0 ml.
* Distilled water	50.0 ml.
Osmium tetroxide (dry)	1.0 gm.
* Sucrose	0.5 gm.
Total amount of solution	69.4 ml.

pH = 7.2 - 7.4
tonicity = 0.3411 M
% O_sO_4 = 1.441

The asterick's indicate the changes. In our laboratory we have found this to yield the best results.

In 1955, Dalton found that the neutralization of potassium dichromate with KOH resulted in the development of a strong buffer system, stable within the pH range of 5.6 to 7.6. Tests with various mixture combinations indicated the most satisfactory combination to be a mixture of 1% dichromate solution (pH 7.2), 1% osmium tetroxide and 0.85% sodium chloride. This combination was obtained by mixing equal quantities of a 4% dichromate solution (brought to pH 7.2 with KOH) and 3.4% sodium chloride. Equal quantities of this resultant solution and 2% osmium tetroxide were then mixed to give the final concentration. Results of the use of this fixation had some advantages and certain disadvantages when compared with the results obtained by Palade's fixative. Some of the advantages were: 1. The very stable nature of the mixture both in regard to pH and spontaneous reduction of osmium. 2. No detectable leaching out of materials during fixation and dehydration. 3. Reduction of damage from polymerization during embedding. 4. Fixation times are not critical. Disadvantages probably related to the advantages were: 1. Lower contrast. 2. Increased difficulty in obtaining thin sections. 3. Mixing of the dichromate solution must be thorough and solution must be at proper pH. This fixative is not widely used at present; but, according to Leon Weiss from John Hopkins, it is felt that it is very applicable to spleen and central nervous system tissue.

In 1956 Luft investigated potassium permanganate for its qualities useful in electron microscopy. He found that the permanganate fixed lipoprotein complexes that appear in membranes especially well, but other parts of cellular structures were fixed poorly. With the background very pale and the membrane density enhanced a very good contrast was produced. He found the potassium salt to be the best applicable and failed to improve fixation by the addition of osmium tetroxide. He felt that permanganate stained glycogen better, and was insoluble in oils and fats in contrast to osmium tetroxide. The method is as follows: 1. Make up the stock solution of potassium permanganate (1.2%), and store in the refrigerator in a stoppered glass. 2. Mix in equal quantities this permanganate solution with acetate-veronal buffer of Palade so that the final concentration is 0.6% potassium permanganate. Buffer pH should equal 7.4 to 7.6. 3. Cool the solution in cracked ice and fix small tissue blocks (1 cmm.) at zero degrees centigrade 15 minutes to 12 hours depending upon the tissue. 4. At end of fixation, rinse the tissue several times in cold (0 to 5° C) 25% ethanol. 5. Allow to warm to room temperature in fresh 25% ethanol (15 to 20 min.). 6. Dehydrate and embed in methacrylate. The temperature here is particularly important. Pease (1960) feels that this solution is hypotonic with resultant swelling of mitocrondria. He also feels that the methacrylate blocks are unduly sensitive to polymerization damage. I feel the biggest problem of Luft's fixative is the loss

of all other structures of the cell. Even when membranes are well fixed and well preserved, orientation is difficult.

Three important factors need mention at this point. First is the time allowed for fixation. (We are here concerning ourselves with fixation by osmium tetroxide.) In our laboratory we are now using fixation times which are individualized to the tissue but usually are between 20 and 30 minutes. Fixation is probably mostly completed within 2 to 3 minutes of proper exposure, and, exposure of more than 2 hours leads to much leaching, precipitation of osmium salts and artifact.

Secondly, is the strength of the osmium tetroxide in the final fixative solution. Palade used 1% in his final solution originally. He has since suggested variations up to 3 to 4%. Others suggest 2%. In our laboratory we have found 1.4% the most useful and convenient. I feel any concentration no less than 1% and no greater than 2% is adequate. Concentrations greater than this are not deleterious, but neither are they an asset.

The third item is temperature control. In chapter two I explained the cooling of the organ in the anesthetized animal. Likewise the optimum action of osmium tetroxide solution is at this temperature for start of fixation. Following the first 3 minutes, the temperature is not so critical, for, as previously mentioned, most fixation has already occurred.

Before leaving fixatives I should like to mention that formalin

fixation can occasionally be used initially, followed by osmium tetroxide staining (fixation?). The results are poor with less well defined borders of structures etc., but it has some use.

According to Dr. J. S. Latta, Dr. W. Bloom, University of Chicago, has had good luck with the fixation of chromosomes with formalin followed by phosphotungstic acid staining. Recently freeze dry and freeze substitution have been invoked in an attempt at eventual histochemical correlation. I have had absolutely no experience with these latter two methods and refer the reader to the works of Sjostrand and Baker (1958) and Ferandez-Moran (1959).

Now that the fixatives used have been discussed, the problem of judging good fixation is at hand. This is judged as the best that can be accomplished by osmium tetroxide fixation. The most conspicuous deficiency in the best preparation is in the preservation of chromosomes. The qualities of good and bad fixation are listed by Pease (1960) as the following: 1. Nuclei uniformly and finely granular. Aggregation of chromatin masses in other than plasma cells (where they are considered normal) is a sign of poor fixation. 2. Mitochondria - (sensitive indicators). Easily become obviously swollen and empty looking while other features are well preserved. 3. Endoplasmic reticulum - appear partly as flattened cisterns arranged in stacks. Width and arrangement of cisterns should be relatively uniform. They become irregularly swollen when poorly preserved. 4. Outer and inner nuclear membrane - the gap between these is a continuation of the cisterns of the endoplasmic reticulum

and, as such, should be of nearly uniform width. 5. Golgi membranes and vacuoles - these seem quite resistant to the problems of fixation and may have a good appearance even though the other organelles are severely damaged. 6. Plasma membrane at the surface of the cell - this should certainly be complete; deep basal infoldings of this membrane should be separated by a nearly uniform gap. 7. The "ground substance" of cytoplasm - this should be very finely precipitated and not at all conspicuous in most cells. Irregular masses of material, coating organelles and inclusions, indicates coarse aggregation of the proteins. 8. Apical ends of secretory cells - these are apt to be more sensitive to deleterious influences than the basal cells. If they seem more watery with their contents widely dispersed by comparison they probably have been swollen. 9. Shrinkage artifacts - these may result in artifical spaces particularly between cells or around inclusions, and, connective tissue is particularly apt to be torn.

Order tends to be degraded by poor preservation. Cytoplasm looks most complex when it is well preserved. Morphological beauty is a correlate of good fixation.

In closing, I would like to once again reemphasize what was said in chapter II. Most of the fixation done in this laboratory, and in most laboratories over the country, is done with osmium tetroxide. This is a dangerous chemical and to prevent injury must be treated as such.

IV.

EMBEDDING

There have been large quantities of work in this field concerning the embedding media for electron microscopy. If one were to take the space in this text to concern himself with all the aspects of this portion of electron microscopy, its purpose would be defeated. However, a basic understanding of some of the essential problems is necessary for the beginner student of electron microscopy. In view of this, an outline will be listed of the principles of embedding with each of the media, followed by a brief discussion of the process. A discussion of polymerization damage will be included at the conclusion of the chapter.

Prior to embedding a tissue block fixed in osmium tetroxide one must concern himself with the process of dehydration. Water contamination in the embedding media leads to artifact in the tissue and bubbles in the block. For all practical purposes the two dehydrating substances primarily used are acetone and alcohol. Dioxane and dimethyl formamide have been tried with no advantages. These latter two chemicals are potentially very dangerous.

The method of dehydration used in our laboratory is as follows:

1.	50% EtOH	15 min.	In Refrigerator
2.	75% EtOH	15 min.	In Refrigerator
3.	95% EtOH	15 min.	
4.	100% EtOH	15 min. X 2 - 4 changes	
5.	50:50 100% EtOH; methacrylate mixture - 30 min.		

The tissue is then continued through the embedding process which will be discussed later.

With the above process we have achieved good results. One can use methyl alcohol for dehydration with somewhat better results, but methyl alcohol is more dangerous. Also, by adding anhydrous calcium sulfate as an indicator in 100% alcohol, one can better regulate the dehydration process.

It is felt by some authors that since ethyl alcohol further reacts with osmium tetroxide, a washing should be done with de-ionized water or a buffered solution, between the steps of fixation and dehydration. Since, however, the reaction is but a continuation of reduction to the lower oxides of osmium, there seems to be no practical value in this procedure.

The use of anhydrous acetone also has a place in electron microscopy. In particular, along with most polyesters, a new polyester "Selectron", recently introduced at the American Association of Anatomists meeting by Low and Clevenger, is not miscible with alcohol. The chief disadvantage of anhydrous acetone is the difficulty in keeping it anhydrous. This occasionally results in incomplete dehydration. An assured way of getting and keeping acetone anhydrous needs yet be perfected.

The author suggests that the use of ethyl alcohol be continued until such time that the less experienced worker wishes to attempt some of the newer embedding media.

Newman et al. (1949) introduced butyl methacrylate embedding. This is the standby embedding media for most beginners. It is also widely used by many experienced workers for most tissues.

In 1958 we were having the same trouble as others in getting blocks of the proper hardness. At that time 100 blocks were prepared with a variance of methyl-butyl methacrylate ratios. These were all prepared in the same manner and were incubated 24 hrs. in a 60° C. oven. Following this, each was sectioned and judged on the basis of hardness, plasticity, homogeneity, and, foremost, on ease of cutting combined with lack of polymerization damage. In conclusion it was found that the mixture of 15% methyl methacrylate and 85% butyl methacrylate plus 1% to 3% by weight of luperco CDB (2,4 dichlorobenzoyl peroxide with a plasticizer dibutyl phthalate) was most advantageous and since then has been used in this laboratory. Other laboratories use anywhere from 10 to 30% methyl or ethyl methacrylate for combining with butyl methacrylate, with good results. The block, when cut with a razor blade, should have a glassy surface, should cut with ease, and should not be so hard or brittle that chunks break off during the trimming process. It is felt in this laboratory and others that if one can indent the embedded block slightly with his fingernail the hardness is probably correct.

As regards the inhibitor (hydroquinone) which comes in the methacrylate, in this laboratory we still feel that this should be removed. By leaving the inhibitor in the methacrylate, the polymerization process is prolonged. The process for removal of the inhibitor is as follows:

1. Fill a separatory funnel 2/3 full of methacrylate and pour in a quantity of 20-30% NaOH (dangerous to eyes, skin and clothing.) - do not fill to top - air space is needed for shaking the solution.

2. Shake separatory funnel vigorously for about 1 minute (cover tip with paper towel) and then replace in support and allow separation of water and methacrylate. The NaOH solution will be brown in color. Open stop-cock and drain off the brown layer.

3. Repeat step #2 until no brown color can be detected (usually 3 times is sufficient.)

4. Drain final NaOH wash and replace with distilled water; shake as before and allow to separate and drain. Repeat this procedure for a total of 6 washings.

5. Filter the methacrylate through two thicknesses of filter paper (place some Drierite in funnel) into a flask containing a layer of Drierite on the bottom. This procedure serves to remove residual water. (about 1%)

6. Place in refrigerator as stock solution and label appropriately - Methyl, Ethyl, or Butyl Methacrylate, purified.

(Caution: Be sure all containers used in the above process are not contaminated by water.)

The outline we use here for embedding following the steps of dehydration and inhibitor removal is:

1. 50:50 100% EtOH : Methyl (15%) - Butyl (85%) Methacrylate mixture-30 min.

2. Pure Methyl (15%) + Butyl (85%) Methacrylate + Luperco CDB 1-2%-30 min.

3. Repeat Step 2 in fresh mixture.

4. Prepolymerize methacrylate by placing in 60° C. oven for about 30 min. depending on consistency of methacrylate as checked by inspection every 5 minutes after the 15 min. period is past.

5. The gelatin capsules (00) are also preheated and coated with prepolymerized methacrylate in the same manner.

6. Place the specimen in the previously prepared gelatin capsules (00) with the prepolymerized methacrylate (with catalyst) 2/3 filling the capsule. Incubate this with the top of the capsule in place 12-24 hours at 60° C. (When the inhibitor is not removed, 48 hours may be required.)

7. Allow to stand at room temperature for 24-48 hours before sectioning.

With incubation in the 60° C. oven, layering of methacrylate with heterogeneity as opposed to homogeneity is still a problem. The purpose of prepolymerization will be discussed later.

In a recent visit to V. L. van Breemen's laboratory, I was able to observe his excellent results with just heating his embedded capsules in an aluminum tray with a regular desk lamp. The aluminum tray had holes the size of the capsules, properly spaced, and the lamp was 6 to 12 inches away from the capsules. This tray allowed proper dispersion of the heat with very little layering. Ultraviolet light has been used in other laboratories in much the same manner with good results.

Methacrylate embedding has many advantages. It decomposes under electron bombardment with resultant loss of substance causing a decrease in the background material and an increase in contrast. This carries with it the main disadvantage of methacrylate, that is, artifact secondary to surface tension factors. This decomposition

causes membranes close to one another to collapse together and appear as a single membrane. "A cylinder may collapse. Two tiny granules that are close together may fuse into a single mass. Structures of complete angular geometry may become rounded. When one is primarily concerned with morphology at the macromolecular level, these artifacts may indeed be serious (although to some extent 'sandwiching' methacrylate sections between two supporting films protects them from this damage....)." (Pease 1960)

The next embedding media is Araldite, an epoxy resin. Maaløe and Birch-Anderson (1956) first achieved partial success with an epoxy resin, which they called EPO, plus an aliphatic polyamine (diethylene triamine) as hardener. Salmonella typhimurium sections were noted to be free of damage and distortion often found in methacrylate embedded sections. Glauert, Rogers and Glauert (1956) next reported success with another epoxy resin known as "Araldite". Following this, Huxley (1957), and Birbeck and Mercer (1957) began a routine use of this substance. Glauert and Glauert (1958) have thus far given the most up to date account in the literature of this substance.

The recommended embedding media mixture is as follows:

Araldite M	10.0 ml.
Hardener 964B (dodecenyl succinic anhydride)	10.0 ml.
Dibutyl phthalate (plasticizer)	1.0 ml.
Accelerator 964C (tridimethylaminomethyl-phenol)	0.5 ml.

(The Araldite M can only be bought from Aero Research Ltd., Duxford, Cambridge, and from Ciba Ltd., Basle. There is no American equiva-

lent of Araldite M. The 964C is sold by Rohm and Haas, Incorporated., Philadelphia. 964B and dibutyl phthalate are readily available.)

Araldite M is miscible in ethanol and the same general plan as used in methacrylate embedding can be used. The exceptions are that the Araldite is more viscous and therefore the mixture is usually heated to 48° C. and soakings and final embedding are carried on at this temperature. Also the time for soaking is longer and varies with the tissue. Typical plans are listed below in a table from Glauert and Glauert (1958). The facts given can be varied to meet the need.

	Soft Specimens Liver, Lung, Spleen, Etc.	Hard Specimens Muscle, Bacteria Etc.
50/50 EtOH/Araldite M	1-2 h. @48°C.	4-6 h. @48°C.
Araldite Mixture less accelerator	2-3 h. @48°C.	24 h. @48°C.
" " with "	2-3 h. @48°C.	36 h. @room temp.

Figure 3

It is advisable to keep separate glassware for epoxy resins and to handle these resins with care, because after polymerization it is impossible to remove these substances. Araldite M, hardener 964B, and dibutyl phthalate will keep for many months at room temperature, but accelerator 964C may deteriorate with time and should be kept as dry as possible. One can best mix resin and hardener at 60° with stirring, and then allow it to stand a few minutes to get rid of any formed air bubbles. This is best done in preheated glassware. The mixture without accelerator will keep for several weeks at zero degrees centigrade. Place the specimen in the bottom of the gelatin capsule

prior to pouring in Araldite. If one needs to replace the specimen, do so with a fine glass rod and take care not to introduce air bubbles.

The blocks of Araldite are light gold in color and show none of the defects associated with methacrylate. Unlike methacrylate, epoxy resins are not degraded under electron bombardment and Araldite sections do not "clear" in the electron beam.

Consequently, the Araldite will not add to the contamination within the microscope. Araldite also appears to have a somewhat greater electron density than methacrylate, but sufficient contrast can be obtained if care is taken to make the sections thin enough.

Dr. J. Luft, as well as Palade and others, uses the "Epon" epoxy resins which are less viscous and less likely to have the "chatter" and "scrapping" artifacts which are present with "Araldite".

Method:

(1) Standard dehydration
(2) Two changes of propylene oxide
(3) Infiltrate for 1 hour in equal parts of propylene oxide and the mixed resin with accelerator.
(4) Finally transferred to the polymerization mixture in gelatin capsules (00) which are heated overnight at 35° C., at 45° C. throughout the next day and at 60° C. during the second night. Cutting is better with aging.

Mixed resin consists of:

2/3 Mixture A + 1/3 mixture B + 1.5% of DMP-30 (Accelerator)

Mixture A (Soft) = Epon 812-62 ml.
DDSA (dodecenyl succinic anhydride) 100 ml.
Mixture B (Hard) = Epon 812-100 ml.
MNA (Methylnadic anhydride) 89 ml.

(Mixtures A & B can be obtained from the Nat'l Aniline Division, Allied Chemical & Dye Corp. 40 Rector St., New York. DMP-30 (tridimethylamino-methylphenol) can be obtained from Rohm & Haas; Wash. Sq.; Phil., Penn.)

The polyesters were first introduced by Kellenberger et al. (1956). A more recent article by Ryter and Kellenberger (1958) gives a good method for the use of Vestopal-W. The polyesters in general are not miscible in alcohol, so acetone is used for dehydration.

Dehydration

30% acetone	15-30 min.
50% acetone	15-30 min.
75% acetone	15-30 min.
90% acetone	30-60 min.
100% (anhydrous) acetone	30-60 min.

Then embed as follows at room temperature:

3 parts 100% acetone and 1 part Vestopal W	-- 30-60 min.
1 part " " and 1 part " "	-- 30-60 min.
1 part " " and 3 parts " "	-- 30-60 min.
Vestopal W + 1% initiator (tertiary butyl perbenzoate) + 0.5% activator (cobalt naphthenate)	-- 30-60 min.

Then polymerize at 60° C. 12-24 hours.

(The Vestopal W and the activator and initiator are available from Martin M. Jaeger, Vesenaz, Geneva, Switzerland.)

The use of Vestopal-W has the advantage of less distortion of the tissue, because, unlike methacrylate, it does not melt but rather sublimates without a surface tension factor. The largest disadvantage is the inability to acquire the substances from the company in a usable state. The initiator and activator are unstable and mixed by themselves may be explosive. It is suggested that benzoyl peroxide and lauryl mercaptan could be employed as initiator and activator respectively. However, these are only partially acceptable.

Vestopal is very hard and tough so that sectioning is difficult and the glass knife must have an angle of 40° to 44° rather than 45° or greater. It is also suggested that this can be helped by the use of dibutyl phthalate as a plasticizer.

A mention here is made of "Selectron," a new epoxy resin manufactured in this country by Pittsburgh Plate Glass Company. It was recently reported by Clevenger and Low, at the annual meeting of American Association of Anatomists (1961), to be miscible with methacrylate and to be useful in electron microscopy as an embedding media. They used it to show what they described as ITM (irregular tubulo-membraneous) substance with great success. Dr. Low readily admits that Clevenger is a very good technician and this substance may not be applicable by all technicians. At this time only limited knowledge is available to the author on this. They dehydrate with acetone and have the following combinations:

50% Selectron 5003 + 50% Butyl Methacrylate
60% Selectron " + 40% " "
40% " " + 60% Selectron 5214
50% " " + 10% " " + 40% Butyl Methacrylate

They found the first mixture in the above listed mixtures to be the most advantageous. The catalyst used was 0.5% tertiary butyl hydroperoxide. They embedded in dry gelatin capsules (00) using a vacuum up to 50 mm. Hg. in one hour, replacing the air with nitrogen slowly (1 hr.). The material was then polymerized in the oven at 60° C. for 12 to 48 hours. It is significant that following the usual osmium tetroxide fixation they had to use 10% formalin treatment

because the "Selectron" would otherwise react with the extra oxygen molecules of the osmium tetroxide.

They found that this was sufficiently strong so that 400-500 mesh nickel grids could be used without previous coating. For better contrast they used heavy metal staining.

Gelatin embedding is used by some for special tissues, but it will only be mentioned here.

Prepolymerization has already been mentioned. This was suggested by Borysko (1956) as a method of preventing polymerization damage. Our method of prepolymerization has already been given. Other methods have been tried in an attempt to eliminate polymerization damage. One was the use of the non-oxidant catalyst, Azodi-iso-butyronitrile, in concentrations of 0.5 to 1.5% with heat polymerization at 47° C. This is useful with tissue cultures, but not with tissue blocks. Ward (1959) has attempted the use of small quantities of uranyl nitrate (0.75-0.37%) added to the methacrylate monomere with some success. This makes the blocks harder and may require the addition of as much as 10% dibutyl phthalate.

What are we trying to prevent by the above modifications? Polymerization damage is likened unto an explosion phenomenon in the tissue primarily due to a differential expansion and contraction during polymerization of certain areas of an embedded tissue block at a given time. It is said that osmium tetroxide itself stimulates the polymerization of methacrylate and that there is more rapid polymerization in the areas around the sites of its activity. The

damage is probably caused by the shearing and unequal tensile forces created by the unequal expansion and contraction. This results in swelling of mitochondria, breaking of membranes, and artifact and distortion sufficient to make small vesicular structures, such as Golgi apparatus and endoplasmic reticulum, unrecognizable. These findings are very difficult to differentiate from fixation artifacts. It is thought that the most problems, as regards, artifacts, arise from polymerization damage.

V.

SECTIONING

Since a primary purpose of this text is its usefulness as a guide to the beginner at this laboratory, I will deal primarily with the equipment in operation here. This means, then that the main emphasis will concern the fundamentals of the sectioning process as carries out on the Porter-Blum Microtome. Some problems of sectioning and its correlated fields will be mentioned, but for a more complete account of these the reader is referred to Pease (1960).

Porter and Blum first described their microtome in 1953 and it was made commercially available that same year. Sjostrand's Model (LKB) was available the same year and is comparably used in Europe. At the recent meeting of the American Association of Anatomists (1961) several automatic ultra microtomes were on display. This included the Fernandez-Moran Model put out by Leitz and a Sjostrand-like machine put out by Philips Electronics. The Philips machine, like the LKB, also had a continuous, persistent cycle. The Leitz machine had a variable cycle and shows promise in the field in the near future. These are all thermal expansion machines except for the Porter-Blum. It has, since its first establishment with the mechanical advancement, consistently given good work. It has very little need of servicing and it is relatively simple to operate.

To continue the processing of tissue in an orderly manner, the step following the completion of polymerization is to remove the

gelatin capsule from around the hardened embedding media. This is done by soaking in lukewarm water for a few minutes and then peeling off the capsule.

For gross trimming the capsule is then placed in the "chuck holder" of the Porter-Blum microtome. (Figures 8 and 9) The larger knurled ring tightens the "chuck holder" onto a special specimen holder. (Figure 7) The smaller knurled ring tightens the "chuck holder" jaws around the embedded methacrylate tissue capsule. One then has a stable capsule for gross trimming. The smaller one can keep the face of the block, the better success one will probably have with sectioning. I usually trim the blocks in a trapezoid fashion as shown in figure 4.

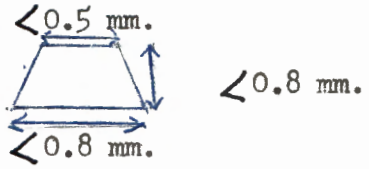


Figure 4.

This has given relatively good results providing one has a well prepared knife. Other authors have stated that a narrower strip is more usable. The reason for this is that if the knife has a few nicks that are relatively widely spaced, one could place this strip between the nicks. This allows one to use the knife if only parts are usable, while the total knife is not. I agree with this, if it is deemed necessary. If the block surface is larger than above specified, one causes a deflection of the knife edge with consequent

digging and skipping, and often a wide variation in section thickness along with the skipping. Even thin sections are apt to be useless because of fine-order chatter.

For the more finite trimming, one can remove the "chuck holder" from the specimen holder by loosening the large knurled ring and attaching it to the cantilever arm on the microtome in the same manner. The cantilever arm lock can then be hooked over the cantilever arm holding the arm firmly in place. Trimming can then be done with the aid of the binocular microscope (BM) mounted on the microtome. The trimming in this laboratory and others is done with a single edged razor blade.

In mounting the capsule it is important to trim the back of the block down sufficiently so that only a very small portion protrudes from the "chuck holder." This provides further safeguard against "chattering," a phenomenon to be explained later. It is also important to orient the block in such a way that the precise tangential section desired is perpendicular to the plane through which the capsule will pass in the sectioning process by the knife.

The next problem is the preparation, selection, and mounting (including trough mounting) of the glass knives. There are various ways of preparing glass knives. In this laboratory a pattern of parallelograms is placed on the desk top. The glass, in a $1\frac{1}{2}$" - 2" x 3/16" - $\frac{1}{2}$" strip is placed over the pattern and with a straight edge a hardened wheel-cutter is used to score the glass. The angle at the breaking edge should be 45° for methacrylate blocks and 42-44° for Epoxy resins and polyesters. The scoring on

the side which is to be used for the knife edge is not extended to the edge. A small horizontal line is then scored near the cutting edge side. A piece of pyrex glass rod is heated to white hot in an O_2-air produced flame and placed about 1/3 to 1/2 of the distance from the cutting edge. The forces of contraction and expansion cause the glass to break along the scored diagonal line with a resultant parallelogram knife. (Figure 12B, Chapter VI)

The knife edge used is indicated in the photograph. One can only judge whether or not a knife is likely to be usable by whether it is relatively straight and whether or not a large "spur" exists. The accompanying photograph taken from Pease's book (1960) shows the portion of the knife which is usually useful. (Figure 10) It also reveals the most common problems encountered. One problem is too large a spur creating too large a nonusable area. Aside from being curved, the spur usually has multiple "checks" on it. These indicate areas which will cause scratches on the section. A second area which cannot be used is that area in which the fracture ridge intersects the knife edge and the area adjacent toward the scored side. The problem this photograph does not show is the tilting of the angle which occurs when the fracture does not follow the predetermined line of etching. This is not a great problem, however, if the edge is otherwise fairly straight and does not have a large spur or too many checks.

We have found that the only real proof of a good knife will be the good sections themselves. However, by proceeding through the

mounting, and creating the combination of a meniscus of fluid, an overhead light and a binocular microscope, we can determine sites of "checking" by the highlights produced. It is very important that the edge of the knife not become contaminated, for this is the seat of most of the problems confronted in sectioning. Before I leave the preparation of knives, I would like to refer the reader to other authors concerning this: Gavin & Lloyd (1959), Pease (1960), Cameron (1956), Tokuyasu & Okamura (1959), and Weiner (1959). Pease (1960) describes a pull, twist and push action in breaking glass knives with a glazier's pliers. In talking with Dr. L. P. Weiss, I was told that a straight pulling action with the glazier's pliers allows a better production of good knives.

Before mounting the knives in the microtome knife holder, a trough is cut out of aluminum from a pattern similar to the following.

Figure 5A

This is then placed with the cut part (arrow) over the top of the long diagonal so that the points curve around to the edge of the knife. (Figure 12B, Chapter VI) A drop of paraffin is placed inside at the base of this trough and allowed to cool. With the trough in perfect alignment, each side is heated with the knife tilted so that the paraffin seals the space between the trough and the knife out to, but not including the edge. I must reemphasize that if the

knife edge becomes contaminated, good sections will be rare. The seal may be reinforced by brushing paraffin along the outside at the knife-trough contact. This seal must be sufficient to hold 10-40% acetone solution in the trough. Others suggest the use of one-half inch tape sealed with filtered beeswax. This has not worked as well in our laboratory but is worth a try by the individual.

One is now ready to mount the knife in the knife holder (B). The positioning of this knife is controlled by the knurled screws (C) and (D). The knife has two short sides and two long sides. One selects the proper point and places that side toward the specimen with the short end nearly vertical to the specimen pathway. When the knife is in place, the angle can be controlled by loosening the knurled screw (A) which allows the knife holder drum to rotate. The proper angle is 1-5° from the vertical. If the angle is greater than this, chattering is often the result and, since this cannot always be noted during sectioning, will often result in loss of good areas detected only at microscopy. Digging and skipping with variance of section size may also result from this. If the angle is too small, or past the vertical, there will be skipping followed by thick sections.

Brief mention must be made here of diamond knives. The author has not used these but plans to do so in the near future. He understands from others that the biggest advantage is that of time saving. The biggest disadvantages are those of multiple scratches on the knife, fragility of the knife, and compression of the sections.

In preparing to section a block, the angle of the knife and the trimming of the block having been accomplished, the operator must bring the knife edge and specimen edge into close proximity. This is necessary in two dimensions. First the horizontal placement of the knife in relation to the specimen may be accomplished by releasing the knife holder by lever (J) allowing it to be moved along a horizontal plane. Once this has been accomplished in line with the specimen the knife can be moved as close as possible by releasing lever (P). Further coarse adjustment can be made by turning knurled screw (M). These are best followed by watching the knife approach the specimen from the side. Following this contact can be made by turning knurled screw (N). The screws (M) and (N) are operated with lever (L) released, which allows smooth advancement of the stage. The first thick sections can be cut by slowly advancing the knife with screw (N). When one finds himself within the tissue block itself, he then can use the automatic fine advance mechanism by setting dial (S) to get the thickness desired. In association with dial (S) is lever (U). This resets the advance mechanism and should be released each time one starts to section.

To advance rapidly at the beginning of a specimen block, one can use the by-pass slide (X). With this the specimen will by-pass the knife for as many cycles as desired yielding a desired thickness of section. For example, if one sets advance dial at 19 (19/40 micron or approximately 1/2 micron) and then bypasses the cycle once, on the second advancement, his section will be approximately 1 micron. This can also be used in obtaining thick sections for light microscopy screening.

The action of the cantilever arm as seen from the front is as shown in the following diagram.

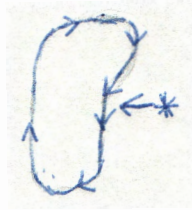


Figure 5B

The asterick indicates where the knife contact is made. This prevents scraping the knife on the way up and prevents wetting the tissue block which would lead to knife contamination. If the specimen block should become wet, one can touch a small piece of filter paper to the edge of the block surface and remove the fluid.

As regards the thickness during sectioning, the Porter-Blum microtome advances on a mechanical basis. The dial setting control is more accurate on this machine that those controlled by thermal expansion. Even so, it too is somewhat fallible. The best way to know section thickness was suggested by Porter and Blum (1953). It was further studied by Peachey (1958). Data from Peachey's laboratory will be correlated with the settings on the advancement control dial of the P-B in the table shown in figure 6. The calculated listings are set forth along with the author's addition of angstrom units, millimicrons and the color scheme of Peachey (1958). In essence, each number on the dial represents the numerator with 40 as the denominator and micron as the unit. The sections approximate these figures, but the color scheme is most accurate.

THICKNESS CONTROL DIAL SETTING	MICRA	MILLIMICRA	COLOR SCHEME	ANGSTROM UNITS
1	1/4		Gray	250
2	2/40	60		500
3	3/40		Silver	750
4	4/40	90		1000
5	5/40		Gold	1250
6	6/40	150		1500
7	7/40		Purple	1750
8	8/40	190		2000
9	9/40		Blue	2250
10	10/40	240		2500
11	11/40		Green	2750
12	12/40	280		3000
13	13/40		Yellow	3250
14	14/40	320		3500
15	15/40			3750
16	16/40		Variable	4000
17	17/40			4250
18	18/40			4500
19	19/40	475		4750

0.001 millimeter = 1.0 micron = 1000.0 millimicra = 10,000.0 Angstrom units

Legend

This table shows the theoretical correlation of Angstrom units, micra, millimicra, thickness control dial setting on the Porter-Blum microtome, and color scheme of Peachey (1958). In practice the best correlation with section thickness is the color scheme.

Figure 6

Ornstein (1956) made some very interesting observations concerning satisfactory sections. He states that there are three demands every useful section must satisfy:

"A. It must be sufficiently transparent to be able to be viewed by transmitted electrons.

B. It must be sufficiently limited in thickness to permit structural analysis in the other two dimensions without confusion of structural overlap.

C. It must be thin enough for the optical system to resolve the structure."

He goes on to say that resolution is the main problem and gives formulae for its calculation. Based on his formulae he gives the maximum thicknesses in angstrom units, that the individual electron microscopes can resolve.

RCA EMU - 2	200
RCA long focus (6)	200
RCA EMU - 3	200 & 800
Philips EM100 (6 mm. bore)	100 & 400
Philips EM100 A&B	300 & 1,200
Siemens ELMISKOP I	200 & 800

The next step in the preparation of the future electron micrograph is an all important one. This is the process of sectioning itself. In the initial process of sectioning, as soon as one has made contact with the block, the trough should be filled with a 10%-40% acetone in water solution for flotation of the cut sections.

One must then continue to cut thick sections until the desired level of the tissue block is reached. This can be checked by removing thick sections on a camel hair brush and placing on an albumin coated slide with a little acetone-water solution present. These can then be fixed with heat and stained for light microscopy or checked by phase-contrast microscopy without staining.

For cutting the thin sections the operator must recognize the possible results of poor operation. Moore, et. a. (1956) wrote a fine article concerning the effects of the microtome knife. They state that the knife produces two main distortions: impact and compression. The impact of the knife blade on the block produces visible distortions of three varieties. "The first is that of vibration of the apparatus due to concussion of the impact; this is of long periodicity, a single period occupying a section. Second, is a wrinkling or rippling of short (30 mm.) periodicity (Williams & Kallman.) Last is a wave distortion of medium periodicity which is due to cutting of compression waves, coming from the initial moment of impact." The above are the various degrees of "chatter" so fluently discussed by Pease (1960). The compression is a grosser distortion caused by the pull of the knife; it causes flattening of the block as with soft paraffin. Another "distortion" is that of the scratches made by nicks in the knife or by a contaminated knife.

To prevent the distortions above and to prevent contamination of the knife edge during sectioning, several observances of the sectioning itself must be considered. The beginner, especially, must recognize

that sectioning is not a rapid process. Nor can it be an irregular, non-attentive process. He must control the moving specimen with a smooth repetitious motion. Two speeds are usually involved. First is the moderately fast "upstroke" as the specimen is returning to its top position. Second is the slow, smooth "downstroke" as the specimen is brought into contact with the knife edge. Cycling the microtome in this fashion, allowing the same amount of time for each part of the cycle, will give a relatively similar good section with each cycle. If the time interval from one "downstroke" to another varies, the variables, such as thermal expansion, with each section will be marked and different, resulting in nonuniformity of sections. Too rapid sectioning is one of the main magnifiers of the "chatter" spoken about in the previous paragraph. The operator should be able to follow the specimen with his eyes as it approaches the knife edge.

The control of the cycle, which must be reestablished each time the operator is forced to stop for a brief period of time, is greatly influenced by the contact the operator has with the machine. I prefer to make contact with the handle of the operating wheel only during the process of sectioning. The manufacturers of our microtome recommend numerous, well distributed, points of contact on the operating wheel during sectioning which I cannot recommend. (See Figure 9) It should be emphasized that any vigorous gripping of the operating wheel or handle creats vibrations which are quickly transmitted to the sections as "chatter." Likewise an unsteady table or unnecessary contact with the machine or table during sectioning will also cause vibration.

I should like to close this chapter by quoting from Pease (1960) a list of common errors that may arise. He set this up as a check list when sections are not being successfully cut.

"1. Knife contamination. This is the most common difficulty, and the fingerprints which produce oily films are the worst offenders. Dirty towels, impure solvents, oily tools, contaminated containers, trough walls--all must be considered.

2. Block face too large. The cutting forces deflect the knife edge with consequent digging and skipping, and often wide variation in section thickness along with the skipping. Even thin sections are apt to be useless because of fine-order chatter. This is second only to contamination as the principal cause of failure.

3. Knife tilted too far forward. This favors digging and subsequent skipping. It is also apt to produce fine chatter which may not be apparent while sectioning is in progress, thus giving a false sense of security.

4. Knife tilted too far backwards. There will be much skipping and occasional thick sections.

5. Cutting speed too fast. This favors fine chatter which may not be apparent during the sectioning. Be sure you can follow the block with your eyes as it descends towards and across the knife.

6. Unsteady cutting. The microtome must be cycled rhythmically;

any interruption or break in this rhythm will require a period of readjustment before good uniform sectioning can begin again.

7. A light touch. The microtome can be deformed enough to ruin quality sectioning simply by a firm grasp on the microtome handle.

8. Meniscus. Too low a meniscus is apt to be the common fault. Back pressures may be created which will prevent the sections from flattening out on the fluid surface as they should. Too high a meniscus is apt to wet the block face which in turn will carry fluid to the knife face, which may result in contamination.

9. Vibrations. The microtome table should be steady. The operator should not touch the table with hands, elbows, or feet while sectioning."

Plates

Chapter V.

Plate II.

Figure 7. Special Specimen Holder used for Gross Training.

(G) Small knurled ring on chuck holder. (Collet-type specimen holder).

(H) Larger knurled ring for adjusting chuck holder.

(I) Special specimen holder of Porter-Blum.

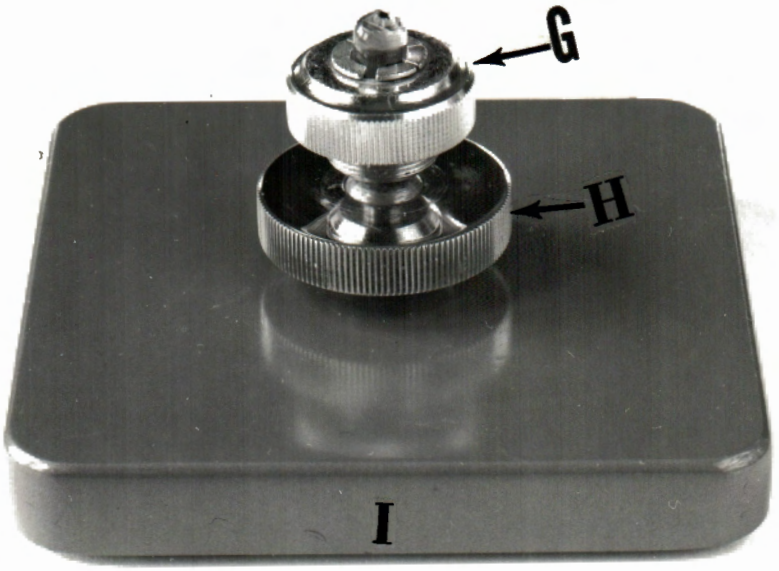

G
H
I

Plate III.

Figure 8. Porter-Blum Microtome.

This is the microtome presently at use in our laboratory. See legend on photograph.

Photograph from the Operation Manual for Porter-Blum Microtome.

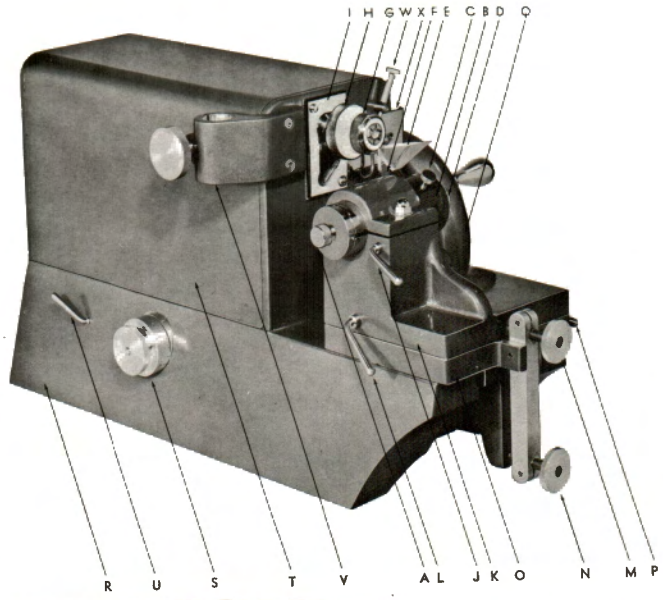
SERVALL®

"PORTER-BLUM" MICROTOME

PARTS DIAGRAM

Patended and Patents Pending

- A Knurled screw for glass knife angle adjustment
- B Glass knife holder with positive angle adjustment
- C Knurled screw for tightening glass knife (F) in knife holder (B)
- D Knurled screw for glass knife width adjustment
- E Aluminum trough
- F Glass knife
- G Collet-type specimen holder
- H Knurled screw for adjusting position of specimen holder (G)
- I Guide plate
- J Lever for releasing glass knife holder
- K Upper stage
- L Lever for releasing upper stage
- M Knurled screw for coarse adjustment of upper stage (K)
- N Knurled screw for fine adjustment of upper stage (K)
- O Lower stage
- P Lever for releasing stage assembly
- Q Operating wheel with handle
- R Base casting
- S Thickness control dial
- T Protective metal hood
- U Lever for resetting advance mechanism after travel forward
- V Bracket for microscope linkage assembly
- W Cantilever arm lock
- X By-pass slide



Ivan Sorvall, Inc. Norwalk, Connecticut, U. S. A.

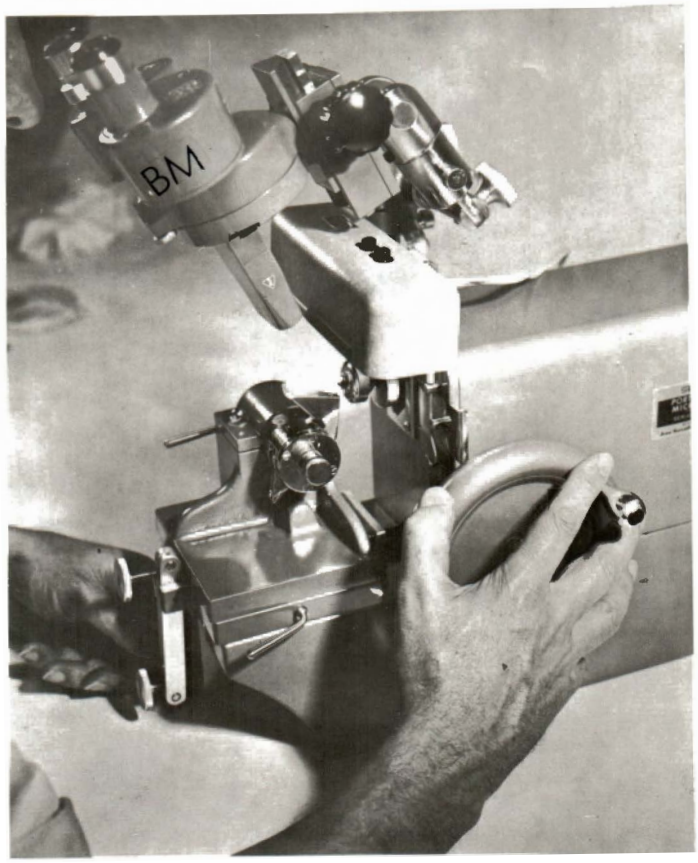
— Designers, Manufacturers and Distributors of "SERVALL" Laboratory Instruments —

Plate IV.

Figure 9. Porter-Blum Microtome with Binocular Microscope attachment (BM).

SERVALL

"PORTER-BLUM" MICROTOME

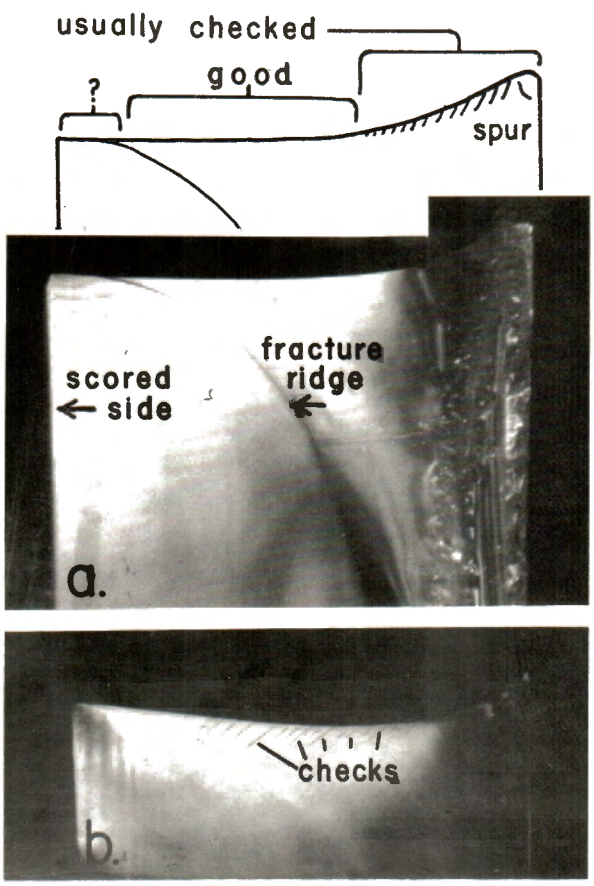


ACT OF SECTIONING — After the trough has been added to the glass knife, and the knife positioned for proper contact with the specimen, sectioning can be started. The force to move the operating wheel is applied at several points, and the rotation is rythmic.

Ivan Sorvall, Inc. NORWALK, CONN.

Plate V.

Figure 10. Glass Knife Defects. (Photograph from Pease, 1960).


usually checked
good
?
spur
scored
side
fracture
ridge
a.
checks
b.

VI.

STAINING-SPECIMEN CARRIERS-SPECIMEN MOUNTING

The first portion of this chapter concerns itself with staining of mounted specimens. The author feels this field still leaves much to be desired. Many experiments by numerous investigators have yielded a minimum of good, reproducible results. The future electron microscopist should keep an alert vigil of the current literature for new successful techniques. For the present three procedural methods will be given and others, with reference, mentioned.

The first method is that used to stain with $KMnO_4$. This substance was mentioned in connection with fixation in chapter III. It stains lipoprotein complexes causing an increase in electron density. The method used in this laboratory adapted from Lawn, 1960, is as follows:

Stain: Dissolve 1 gm. of $KMnO_4$ in 100 ml. distilled water. Do not filter. Allow to stand at least 24 hours before use.

Procedure:

1. Fill a small vessel with staining solution removed with a pipette from beneath the surface film in the stock bottle.
2. Sweep the surface of the staining solution with the edge of a glass slide coated with paraffin wax.
3. Float thin sections (without removal of the resin), already mounted, section side down on the surface of

the staining solution, immediately after the surface has been cleaned. Stain for 10 minutes to 2 hours. (10-20 minutes usually sufficient)

4. Rinse in running water (distilled) for a few seconds.
5. Immerse and agitate in a solution of 1 drop of 5% citric acid in 1 ml. distilled water for 30 seconds to 1 minute.
6. Rinse in distilled water.
7. Dry on filter paper.

(Time of staining varies with fixation)

A second method, staining with phosphotungstic acid, adapted from Hall, et. al., (1945), is shown in the following procedure:

1. Float mounted sections face down on a 25% alcoholic solution of phosphotungstic acid for 10-15 minutes.
2. Remove and wash in distilled water.
3. Dry on filter paper.

The third method mentioned is that of Dalton & Ziegel, 1960. This is lead acetate staining. The procedure is as follows.

1. Place small amount of saturated Pb acetate solution on a clean slide.
2. Place mounted sections face down on this solution for 10-15 minutes.
3. Remove and dry by placing face up on filter paper.
4. Hold over a 5% (or greater) solution of ammonium hydroxide for 5 to 10 seconds.

In addition to the above stains, Watson (1958) suggests the use of other heavy metals in staining. An excellent article on staining of methacrylate embedded tissue was written by Houck and Dempsey (1954). The stains listed above are those proven to be most adaptable at the present time. More techniques are being tried throughout the country and will surely be available in the near future. It is hoped that until then, these will satisfy the various needs.

There are two general types of specimen carriers, the round mesh grids and the grids with a rectangular hole in the middle. It is probably best to start with the mesh grids which range from 200-500 mesh, copper (C) or nickle (N). (Figure II) There is a convex and a concave side which should be identified before coating with a film. The only disadvantage of these grids as compared to the others is that of seeing all areas of specimen. It invariably happens that the part of a specimen that is of most interest is behind the bar of the grid.

The second type specimen carrier has a larger rectangular hole in the middle. (Figure 11P) This is a Philips specimen carrier and has a shiny and a dull side. This carrier is said to cause undue difficulty in picking up a specimen over the proper space. This is not a problem after one has some practice, and the advantage of the large viewing area far outweighs any disadvantage that might be present at the beginning.

The next concern is the preparation of grids. The method most used in this laboratory is that of formvar coating, and more recently,

stabilization of these grids with a carbon film.

The most important aspects of a film coating are: 1. A sufficiently thin coating to allow good contrast of the specimen itself. 2. Strength sufficient to give good support to the specimen itself. 3. Stability sufficient to prevent drift of the specimen during electron bombardment. 4. A coating free from contamination so that important structures will not be obscured.

The formvar we use satisfies all but the stability requirement. It allows drifting of the specimen making it, used alone, less satisfactory.

The procedure is:

1. Fill a glass container with water and sweep the surface clean with lens tissue just before use.
2. Dip a clean side into 0.1% Formvar solution in ethylene dichloride and put out and allow to drain and dry slowly.
3. Score film at the end of the slide and along the sides where it is to separate from the slide. Hold slide in front of mouth and frost it by blowing.
4. Insert under the surface of the water at an angle, varying with the method the operator is accustomed to. Most use 30°; others claim one can dip slide in vertically and get two films at once. Watch for the film to separate, with only the thicker end being visible as a grayish membrane. The thin end should be visible only at the sides.
5. If the membrane appears to be too thick, hold slide in

ethylene dichloride vapor for 10-20 seconds before drying the next formvar film.

6. Pick up single grids from the box. If it is the mesh type place the convex surface down onto the film. If it is the Philips type, place the shiny side down on the film.
7. Place the grids with some space between them avoiding the extreme edges and the far end of the film.
8. Use no more than the thinnest one-third of the film.
9. Using a dry slide, pick up the grids by bringing the slide down in a horizontal position onto the grids and immersing grids and film into the water. Then turn the slide to the side and upwards so that the grid surface is on top when one emerges from the water. The grids will now have a thin coating of formvar on their convex or shiny side.
10. Dry the grids in the bottom of a petri dish covered by filter paper. When they are dried, place them in a covered petri dish with filter paper on the bottom.
11. If one is not sure that the grids are properly covered, it is best to discard them and try again. Much time can be lost if poorly covered grids are used.

Parlodion films are made in much the same manner here using a 0.5-1.0% solution in amyl acetate. Some feel that the parlodion film is easier to make. The rest of the procedure is essentially the same.

For carbon stabilization one uses a vacuum machine in which carbon evaporation is produced. Two small-tipped carbon rods are held

against one another and a current of high amperage and low voltage is passed through the system. This causes evaporation of carbon onto the grids creating a fine carbon film over the parlodion or formvar film. This serves to stabilize and increase support of the film. This prevents drift, the most important advantage of this procedure. It does decrease contrast, but not sufficiently to create a distinct disadvantage if properly controlled. For details of running the carbon evaporation machine, the reader is referred to the operating manual sent with the machine by each company.

Carbon coating by itself can be used and is accomplished in the same way with a somewhat thicker layer of carbon applied. It is also possible to dissolve out the previously described parlodion or formvar films with the appropriate solvent. This leaves a single layer of carbon film.

Dr. V. L. van Breemen (personal communication) has constructed an apparatus which allows several slides of grids to be coated at once. This is a half-moon shaped metal apparatus with holders on the inside for the slides. This fits in the vacuum evaporator with the carbon rods at the mid-point of the diameter of this semi-circle, and the slides, with grids, each equidistant from the center. This adds greatly to the efficiency of this process.

Now that the grids have been properly prepared and the staining procedures have been given, it seems appropriate that the mounting of the specimen be discussed.

Following cutting of the specimen, the sections float on the

acetone-water solution in the trough. Those that are of no use may be removed with a small camel-hair brush and discarded by lightly rubbing sections off the brush and onto clean filter paper. According to Peachey & Satir, (1958), as the sections float out onto the acetone-H_2O bath, they are 30-50% compressed. If left alone, these sections will reexpand 1/4 of that compression. Heat will increase this return toward original size, but also has a thermal effect on the microtome. Xylol fumes will return these sections to 85-90% of normal. There is a danger of overexpansion if the xylol is held too close to the section or for too long. A glass or wooden rod dipped in Xylol and then waved slowly across the sections floating in the trough, is very effective.

When good sections are ready for mounting, one should pick up the grid on one end with a pair of fine forceps. (Figure 12C) Enter the trough solution and, with the aid of the binocular microscope, slowly advance the grid under the desired sections, slightly "overshooting" the area wanted. Rapidly bring the grid straight up and out of the solution. Due to surface tension factors, the section will slide forward a small distance and will settle itself precisely in the location one had desired. One can use this for both the Philips and mesh type grids, and I have encountered very little difficulty with the former. One should check with a light microscope to make sure he has the specimen in place in the doubtful ones.

The grids are then dried by placing on filter paper and in 10-15 minutes are ready to use. If they are not used immediately, they can

be stored in a covered petri dish with filter paper on the bottom. A special holder, constructed by the author, is shown in figure 12-A. With this, one has numbered rectangular slots in which the grids can be stored with proper identification. The long area at the edge is an area where the grids may be dried and transported, if immediate viewing is anticipated. This holder was constructed from plastic. The lid prevents contamination and the holder is a convenient way of storing and transporting mounted specimens.

It is evident that technique of preparation of specimens for the electron microscope is no small undertaking. Perfection is possible only after much practice. These chapters are far from complete, but the author hopes that these will serve as a guide to those interested in preparing specimens for electron microscopy. He also hopes, that in the not too distant future, these chapters can be expanded and revised into an even more useful tool for many. Next--to microscopy itself.

Plates

Chapter VI.

Plate VI.

Figure 11. Specimen Carriers (Holders or Grids).

(C) Copper mesh specimen carrier.

(P) Philips' specimen carrier.

(N) Nickel mesh specimen carrier.

(Magnification 3X)

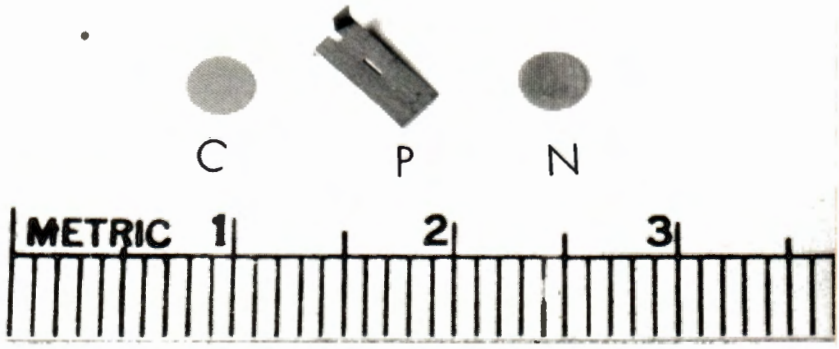

C
P
N
METRIC 1
2
3

Plate VII.

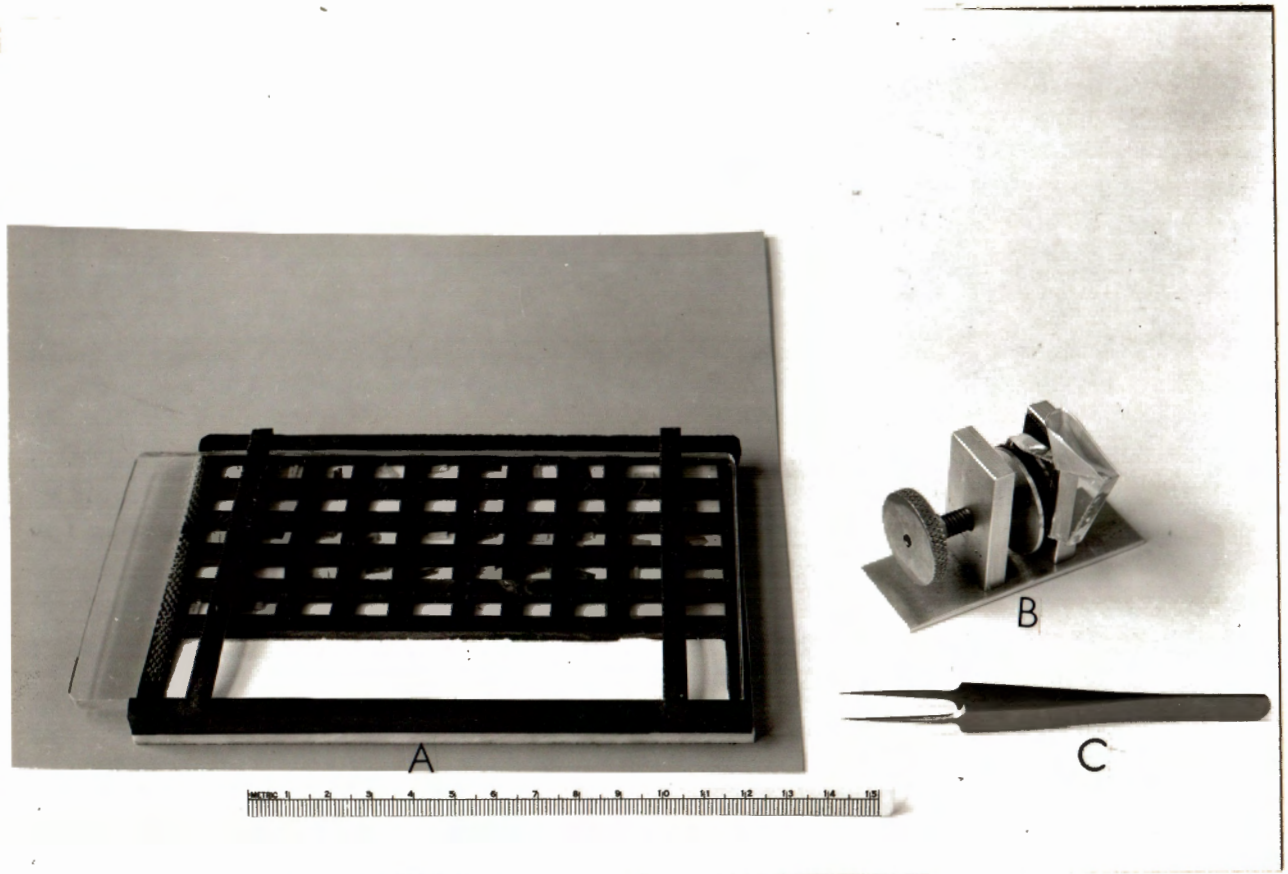
Figure 12. Special Adaptations for Electron Microscopy.

(A) A special specimen transportation and storage holder.

(B) Knife with trough held by special knife holder originally designed to allow viewing of knife edge. No longer in use at this laboratory.

(C) Fine, well-constructed forceps used for controlled transfer of grids.

(A & B constructed at this laboratory).


A
B
C

VII.

THE ELECTRON MICROSCOPE

Since the invention of the compound microscope by Zaccharias Jansen in Holland and Galileo in Italy simultaneously in 1609, there have been multiple names associated with microscopy. (Zinser, 1960) Hooke, 1663, and van Leeuwenhoek, 1673, are names familiar to all. In 1854, Jabez Hogg, an eminent London ophthalmic surgeon, stated that he foresaw the general acceptance of the microscope as an indispensable tool of medicine. The "Microscopical Society of London" was formed in 1839, and was the coordinating center of the great development of the microscope in the 19th century. J. J. Lister in 1860 first combined the objective and the ocular in a microscope, but it was not useful at high power. Carl Zeiss, a microscope maker, in 1866 employed the mathematician Ernst Abbe. The latter made about the last major step in light microscopy by constructing new perfectly designed "Apochromats". This made the fundamental laws of nature, rather than shortcomings in design, the limiting factor in the resolving power of microscopes. (Jansen, 1960)

Due to the wave length of light, the best resolving power with a light microscope is 1000 A°. The wave length of light could not be reduced. Ultra violet radiation was awkward to use. Radiowaves were not useful because their lengths were tens of thousands of times as long as those of light. X-ray wave length was short enough, but no realization of their control was then known and their destructive action on the minute structures and organisms was too well known.

In 1924, de Broglie formulated the hypothesis, which was later proven, that a beam of electrons should under certain circumstances be considered as radiation with the character of waves. This created interest because an "optical" system for electron rays was a practical possibility. (Jansen, 1960)

It was found that the wave length of electron beams was small enough to promise a considerable advantage over light if a microscope could be constructed on the basis of the use of electrons as the medium for image formation. About 1930, Knoll and Rushka, in Germany, proposed to build such a microscope and proceeded to do so with overwhelming success. We thus had a machine capable of very fine definition by employing rather small angles of aperture and a very small wave length. The early fears of destruction and distortion of tissue by vacuum and electron bombardment did not prove to be problems. Multiple improvements have occurred since that time and shall continue to occur. Today we have several companies making Electron microscopes. Those most in use in the United States today are the RCA, the Siemens, and the Philips microscopes. The Siemens and the Philips EM 200, are capable of a resolving power of 5-10 A°, almost 200 X that of the ordinary light microscope. This yields magnifications in the neighborhood of 200,000 diameter at the screen. In our laboratory we have an EM 100 B which is capable of 15-20 A° resolution and 100,000 diameter magnification at the screen, with photographic enlargement up to 200,000 to 250,000 diameters. The scientists of this century are, indeed, greatly indebted to those

before us who have provided us with such magnificent tools to explore the macromolecular universe.

VIII.

PRACTICAL THEORY OF THE ELECTRON MICROSCOPE

The electron microscope is an enormous maze of wires, tubes, pumps, and adjustments. Its complete explanation is far beyond the scope of this book and falls in the realm of the electrical engineers who service the machine. Therefore, I will attempt only to give a basic theoretical background in this chapter.

The first consideration is to understand the superficial mechanics of an electrical lens system. In order to do this, we must first somewhat understand the principle of the electrical magnetic lens (L1-L5) and then apply this to the governing of electron beams from the electron gun (C). (See Figures 13 & 14)

The electro magnetic lens (a. without and b. with pole pieces) creates around it a magnetic field indicated by the magnetic lines of force (F). The lens itself is a coil of wire enclosed in an iron jacket. (S) is the slit in the magnetic circuit and (P) the pole pieces. The object is at (V), the image is at (B). The general principle is that the electron beam is altered in direction depending on the speed, direction and size of the electron as influenced by the magnetic lines in the magnetic field. The function of the pole pieces, as can be seen by figure 13 is to change the focal length. In (a.) it may be several centimeters, while in (b.), a few millimeters. These lenses control the trajectory pathways of the electron beam.

When one correlates these into the vacuum system of an electron microscope, figure 14 helps explain what happens. This is a cross-sectional view of the Philips EM 100 B. This magnetic electron microscope is built by the Philips concern in Holland after a model developed by Le Poole. The column is almost horizontal, and the large fluorescent screen is well situated for convenient viewing. The essential parts consist of:

L1	-	condenser lens
L2	-	objective
L3	-	diffraction lens
L4	-	intermediate lens
L5	-	projector
C	-	the electron gun
S	-	fluorescent screen
Top Arrow	-	indicates specimen & direction

By the use of the four magnifying lenses (L_2-L_5), a very large range of magnification can be covered, and diffraction patterns of crystalline specimens can be focused at the final screen while the object is in the usual position for microscopy. As shown in the first diagram, with lenses L_2, L_4 and L_5 magnifying in series (i.e. with the diffraction lens not energized), a situation with a high magnification range can be covered (4000 - 60,000 X at the screen). In the middle diagram the intermediate lens (L_4) is off and with the diffraction lens (L3 - a weak lens of large bore) demagnifying the image from the objective (L2), the low range from 1000X to 4000X can be covered. As shown in the 3rd diagram, the lenses L_2, L_3 and L_5 can also be activated to focus diffraction patterns of the specimen on the final screen. The beam potential may be set at 40, 60, 80, and 100 KV.

Unlike the objective in most magnetic instruments, the objective lens (L_2) has a large gap and bore diameter, both of which dimensions are about 10 mm. Consequently, machining tolerances and inhomogenities in the iron are likely to produce less distortion of the field near the axis than is the case when these dimensions are small. In order to achieve the relatively short focal length of about 4.5 mm. with a field of such a large axial extent, the object is situated well inside the field between the faces of the pole pieces, which permits a position of relatively simple specimen-insertion mechanism. The specimen is mounted on a rod and inserted with a straight thrust through rubber seals into the magnetic gap. The amount of air taken in with a specimen is so small that reevacuation of the tube requires only about 20 seconds. Similarly, objective apertures of several sizes ride on a rod in the gap of the objective, where they can be aligned or exchanged while the instrument is in operation and easily withdrawn from the instrument for cleaning or replacement. A disadvantage of this type of construction is that the lenses cannot be compensated to allow for field asymmetries, although the possibilities of these occurring should be less with the large bore. The lens assembly is rigidly constructed without alignment screws accessible to the operator since it is assumed that the alignment made in manufacture and installation is adequate.

An interesting device first introduced by Le Poole as an aid to focusing is situated above the objective lens. The beam passes through the fields from two sets of magnetic-deflection coils set in opposite

polarity. When the coils are energized, the beam is deflected away from the axis by the first coils and back with the second so that the illuminating pencil strikes the object in the same place, but at an angle to the axis. In normal operation the coils are off, but as an aid to focusing, they carry current at 50 cps., causing the inclination of the beam to vary between extremes. When the objective is focused on either side of the specimen plane, the image oscillates on the final screen. In effect, the device increases the relative aperture of illumination, which results in a greater blurring of the image with defocusing, (the wobbler system). Thus, if the image is too faint or cannot be seen clearly for other reasons, it is still easily possible to set the objective-current control at the point for least image sweep.

Micrographs are recorded on 35 mm. film with the camera placed close to the final projection lens. As many as 20 exposures can be recorded on one strip, but the magnification of the recorded image is only about one fourth (1:3.5) that on the final screen. To record micrographs at 15,000X, for example, requires a 60,000X on the screen, at which magnification the intensity is too low for satisfactory observation. The 35 mm. roll film is used for scanning. A plate camera mounted on front allows better resolution at high powers. The outgassing of film takes an inordinately long time that may, however, be reduced by prepumping for long periods in an auxilliary vacuum chamber.

While the instrument is in operation, the diffusion-pump system, consisting of an oil pump and a mercury pump in series, works into a

buffer tank with the mechanical pump off to reduce vibration. The power consumption is 2.5 to 3 kw. Although the input circuits are designed to connect to a type of three-phase, four-wire line not common in this country, conversion transformers are available for operation from conventional three-phase 220-volt lines such as we use in Nebraska. Water requirements for cooling the lenses, mercury pump, and oil-diffusion pump are 2.5 qt/min at 20 psi pressure and a temperature not in excess of 60° F. (Hall, 1953 and Jansen, 1960)

So much for the theoretical aspects of the electron microscope.

Plates

Chapter VIII.

Plate VIII.

Figure 13. Electro-Magnetic Lens. (Photograph from Jansen, P. C. 1960).

(a) Electro-magnetic lens without pole pieces.

(b) Electro-magnetic lens with pole pieces.

(F) Magnetic lines of force.

(S) Slit in the magnetic circuit.

(P) The pole area.

(V) Object.

(B) Image.

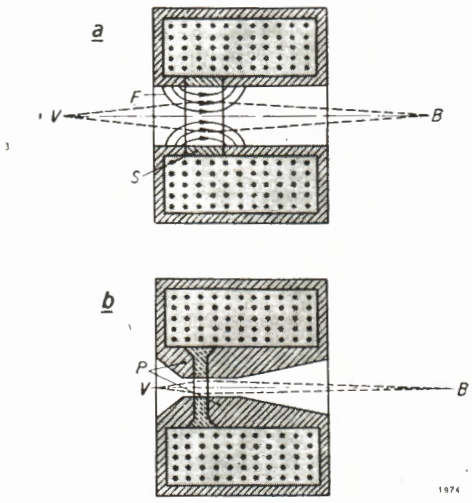

a
F
V
B
S
b
P
V
B
1974

Plate IX.

Figure 14. Cross-section Schematic Representation of Electron Paths in the Philips Electron Microscope.

(L1) Condenser lens.

(L2) Objective.

(L3) Diffraction lens.

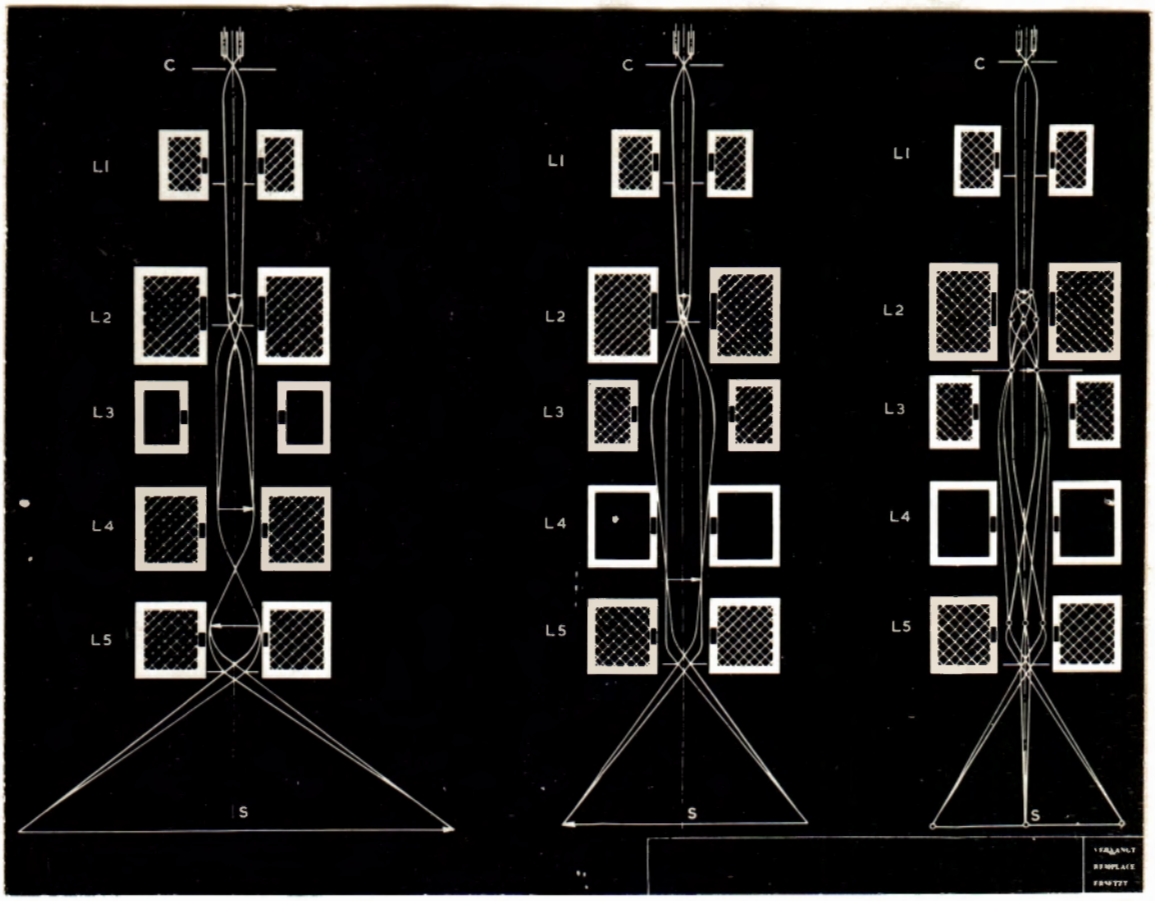
(L4) Intermediate lens.

(L5) Projector.

(C) Electron gun.

(S) Fluorescent screen.

(Top Arrow) Indicates specimen and direction.


C
L1
L2
L3
L4
L5
S
C
L1
L2
L3
L4
L5
S
C
L1
L2
L3
L4
L5
S

IX.

OPERATION OF THE PHILIPS ELECTRON MICROSCOPE-100B

It is obvious that the many problems that arise during the operation of this machine cannot be handled on paper; but, rather, can be worked out by the individual as he gains experience. The basic operation of the microscope and a few of the associated problems will be superficially discussed. The reader is referred to the operation manual printed by the Philips Electronics Company for a better understanding of its intricate operative mechanisms.

Before reading the following instructions the reader should familiarize himself with the parts of the machine. This can be accomplished by turning to the last few pages of the chapter and correlating the legend and the photographs. (Figures 15-17)

Putting the machine into operation requires following rigidly a specific technique. The rear flap and the covering plates of the microscope tube should be closed. Knob (W3) of the valve box should be in neutral position with the high tension switch (W1) at 80 K.V. Turn the condenser-lens current knob (R617) toward the front of the machine as far as it will turn decreasing the current to its minimum. Likewise, the filament-adjustment knob (R601) should be turned toward the operator as far as possible (press and turn) to keep heater current at its minimum. The camera should be in place with the cover (K10) closed and there, theoretically, should be no specimen holder (K12) in place. Practically, this latter requirement is often overlooked without serious results. By turning knob (K6) clockwise until

it "clicks" the camera is placed out of the field of the electron beam. By turning knob (K9) away from himself as far as possible, the operator places the small objective aperture in the beam. Knobs (K7a) and (K8a) adjust the diffraction diaphragms and should be adjusted so that they are not in the beam pathway.

After the above settings have been checked, one can open the tap-water cock sufficiently to energize the water switch as indicated by a clicking of the microswitch. Switch on the master mains switch. This will be followed by the lighting of tube (L7) and, the correlated vacuum meter (M2) will be in the red, both of which indicate the degree of vacuum. At this position insufficient vacuum is present. Later in the operation the vacuum indicating tube (L7) will be nearly extinguished and the vacuum meter (M2) will show the pointer in the green part of the scale. This means that the space to which the "Penning" vacuum gauge mechanism is connected, has reached a deep vacuum.

Next press push-button (D2) which turns on the lens currents and begin vacuum production. This is accomplished in several steps.

1. Turn knob (W3) into position "half." This evacuates the connection between the pre-vacuum pump and the valve-box.
2. In approximately 10 seconds turn knob (W3) to position "2" via position "1." The buffer tank is now evacuated. Leave the knob in this position until the valve in the pre-vacuum pump makes a sharp chattering noise.

   (During the several pumping stages the valve, which is situated in the pump, makes a characteristic sound. If

the sound is of a high, dry pitch, the space, which is connected with the pump is well evacuated.)

3. Turn knob (W3) back to position "1". The tube is now evacuated. Leave the knob in this position until the valve in the prevacuum pump starts chattering sharply again.
(If the sharp chattering noise of the valve in positions "1" and "2" are not the same there is probably a leak in the pump unit or in the microscope column, which must be eliminated first.)

4. When the vacuum indicating tube (L7) and the vacuum meter (M2) are nearly extinguished and in the green respectively, the high vacuum pumps are operating properly and knob (W3) may be turned to position "3". This includes the microscope tube in the vacuum system thus depleting the vacuum and (L7) and (M2) will immediately indicate this.

5. When the vacuum is shown again to be sufficient by (L7) and (M2) the knob (W3) is turned to position "4" taking the prevacuum pump out of the system and this is shut off.
"It is of the utmost importance to keep strictly to the order in which the foregoing manipulations are given, because if the microscope tube is not properly pre-evacuated before the high-vacuum pumps are started up, these may be thrown out of order and the apparatus will have to be stopped for some length of time to give the pumps an opportunity to be regenerated. There is also the risk of oil vapour getting into the

microscope tube, condensing inside it and setting up static charges interfering with the picture; the oil can only be removed after dismantling the microscope tube." (Philips Electronics Operation Manual)

When a sufficiently high vacuum in the microscope column is reached, as is indicated by the lighting of 1/2 of tube (L7) and by the meter (M2) pointer in the green, the high tension may be switched on by pressing button (D-5). This lights up the indicator lamp (L604).

Now increase the heating voltage by turning knob (R601) away from the front of the machine after depressing it. This increases the emission current of the microscope column adjusting this to 20uA as indicated on meter (M4). By the use of knobs (W2) and (R623) adjust magnification to approximately 3,000 X. A light will now appear on the screen. If it does not, one should readjust the currents of the deflecting system via knobs (R612) and (R613) in such a way that light does appear on the screen. (Note: R612 and R613 usually need only a small amount of turning.)

Turn the condenser lens knob (R617) away from the front of the machine so that maximum light appears on the screen. Again knobs (R612) and (R613) may necessarily be used to keep light on the screen. Increase the heating current via knob (R601) until the meter (M1) moves to the position of the first arrow. The microscope is now ready for use.

The placing of the specimen in the tube is next on the agenda. If this has already been done, these instructions will still serve each time a change is made. It is obvious that, first, one must put

the specimen carrier into its specimen holder. (Figure 17) To do this when using the Philips specimen carrier (9) one presses cam (7) on specimen holder (B), and moves it into the direction of the knob. With the aid of the tweezers the specimen carrier (9), with the shiny side (specimen side) up, is placed in the slit of the holder and cam (7) is allowed to jump back into its original position. If one is using a copper or nickel mesh grid (2) loosen screw (4), on specimen holder (A), one or two turns and slide spring (C) outwards with a slight lifting motion. Then place grid (2) convex (specimen) side up, over the opening in the holder. Return spring to original position and tighten screw (4).

Before placing specimen in the microscope, be sure that the high tension is off by pressing button (D4). Turn knobs (R601) and (R617) maximally toward the front of the machine, adjusting heater current and condenser lens to a minimum.

Then place the specimen holder in the microscope tube with Pin (6) sliding into the anterior acceptance groove. A small amount of air will enter into the tube when the specimen holder is inserted resulting in a temporary insufficiency of the vacuum. Tube (L7) and meter (M-2) will show this. After approximately 20 seconds the vacuum will have regained its original value and operation can be resumed.

Now switch on the high tension by pressing knob (R601) and turning away from the front of the machine so that (M4) reads up to 20uA, set the magnification at approximately 4,000X with the aid of the meter M3, knobs (R623) and (W2), and the magnification scale in the operators manual. If the screen does not light up the specimen is probably not

in the electron beam and can be returned to proper position by the use of knobs (K1) and (K2). Increase the filament current by knob (R601) to the first arrow on the meter (M1), and then by manipulation of knobs (W2) and (R623) and, according to the operator's wishes, set the magnification required.

Focus as much as possible with the aid of the objective lens (knob R640). For fine focus combine the objective (R640, and R641) and the wobbler system. The latter is controlled by a floor button which is held depressed while focusing is perfected with the aid of knob (R640) and a binocular microscope. If another K.V. is selected, refocusing will be necessary. This gives one a formed image.

During the use of the microscope a combination of various manipulations will be necessary to obtain the proper field, magnification, focus and contrast. These include the use of knobs (W2) and (R623) for magnification, (K1), (K2) and a floor button (the scanning device, allowing greater area to be seen) for scanning tissue, n the condenser current knob (R617) to vary illumination and contrast. The different size of the objective diaphragm as governed by knob (K9) will be used for finer detail and better contrast in taking pictures.

Following the previous process of focus and adjustment, during which the proper specimen area, focus, and magnification have been obtained, one is ready to take electron micrographs. These are, of course, taken with the objective diaphragm engaged. This is accomplished by turning knob (K9) toward the back of the machine as far as possible. Caution: when turning this knob toward the front of the machine, do not turn farther than is necessary

to cause complete illumination of the screen, as it can be screwed out far enough to allow an inrush of air, ruining the vacuum and contaminating the machine.

To indicate the distance of a micron on the photograph or the screen, move, via knob (K5) the movable wire to the proper number of the scale on the screen. This is correlated with the known magnification divided by 1,000. (e.g. For magnification 30,000 use scale number 30.)

Next one should determine the exposure time which is dependent on the adjustment of the condenser lens, on the magnification, on the film used and on the film developing method. This obviously is different in each laboratory and the particular method should be learned as indicated.

To bring the camera into the beam, turn knob (K6) fully in the direction toward the front of the microscope after pressing button (K17) which holds the camera in place out of position. The shutter of the camera is operated by knob (K4). This knob should be brought quickly forward for the proper exposure time and then quickly returned to the other position. If this is not done with a rapid motion, parts of the film will be less exposed than others.

The camera is then returned to its original position by turning knob (K6) away from the front of the microscope until button (K17) clicks in stop position. It is significant to note that the magnification at the film is about 1/4 that of the magnification at the screen, but photographic enlargement can compensate for this. (Actual

ratio equals 1:3.5). The front plate camera has only recently been put into use at our laboratory. I am not familiar with the intricate mechanisms of the front plate camera, but recognize its distinct advantage at high resolution and will soon become so, as should the reader.

Logically, here should be inserted a small section concerning the vacuum during operation of the microscope. As successive specimens are exchanged, small amounts of air enter the microscope column causing depletion of the vacuum in the buffer tank. Correspondingly, the deep vacuum will be lost, as indicated by tube (L7) and meter (M2). If the vacuum in the buffer tank becomes very low, t e red lamp (L605) will start burning. (This also indicates problems in the cooling system.)

To re-evacuate the tank, turn knob (W3) from position "4" to "3½". This will switch on the prevacuum pump. This will usually begin with a muddy sound rather than a sharp chattering one. The latter, however, will appear in about 30 seconds and following this (W3) should be turned to position "3".

IMPORTANT:

> "If knob (W3) is turned straight from position "4" to position "3", the air contained in the pipe connecting the pre-vacuum pump to the valve box will flow into the buffer tank and the high-vacuum pump. The result is a throwing out of order of the high-vacuum pumps."
> (Philips Electronics Operation Manual)

When the pre-vacuum pump starts to chatter sharply, knob (W3) can be turned to position "4".

When the microscope is to be put out of operation the knob (W3) is turned clockwise from position "4" to position "0". The master mains switch is then turned off. Allowing 30 minutes for the vacuum unit to cool with the water running through it, one may then turn off the water. A fan may accelerate this latter step.

These are the basic steps in operating the Philips EM 100B. The student should familiarize himself with the various knobs and meters on the machine. He should then thoroughly study the plan of operation. With an experienced worker, he should observe and eventually "solo" through the technique under observation. Before operating the maching without observation, the student should have observed a sufficient amount of time to be familiar with the many problems that can occur during operation and the plan of "trouble shooting" when they do.

Plates

Chapter IX

Plates X, XI, XII.

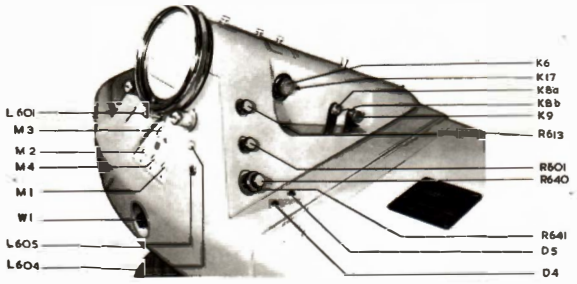
Figures 15-17. Several Views of the Philips Electron Microscope 100B. (Photographs from Philips' Electronic Manual).

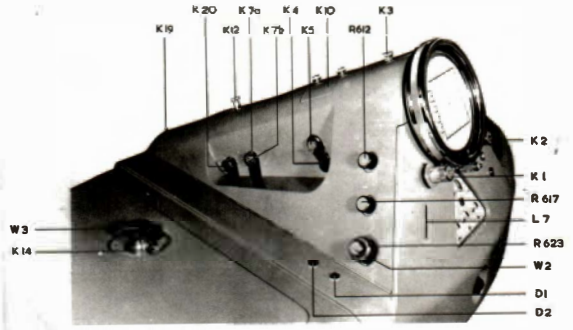
(D1) Push-button for switching off the lenses.
(D2) Push-button for switching on the lenses.
(D3) Foot-switch for switching on the focusing system (Wobbler).
(D4) Push-button for switching off the high tension.
(D5) Push-button for switching on the high tension.
(K1, K2) Knobs by which the specimen can be moved.
(K3) Lock for locking the covers of the microscope tube.
(K4) Knobs by which the camera shutter is controlled.
(K5) Knobs for adjecting the micron-marker.
(K6) Knobs by which the camera can be moved into the electron beam.
(K7a,K7b K8a,K8b) Knobs by which the plates of the diffraction-diaphragms can be adjusted.
(K9) Knob for selecting the objective-diaphragm.
(K10) Cover of the camera-opening of the microscope tube.
(K12) Specimen-holder.
(K13) Crank handle by which the microscope tube can be moved upwards.
(K14) Knob for controlling the air inlet valve of the microscope column.
(K17) Stop of camera knob K6.
(K19) Lock of the rear-flap.
(K20) Knob for setting the Stigmator.
(L7) Indication lamp for control of the high vacuum.
(L601) Exposure meter.
(L604) White signal-lamp; when burning it indicates that the high tension is switched on.
(L605) Red signal lamp; when it burns it indicates that the apparatus is not ready for use for one of the following reasons:
a. an insufficient quantity of water flows through the cooling system.
b. the vacuum in the buffer tank is insufficient.
(M1) Meter by which the filament voltage of the microscope tube can be checked.
(M2) Vacuum indication meter.
(M3) Magnification meter. This meter is used in combination with the magnification scales. See also under W2.

PHILIPS
EINDHOVEN
SERV. MED. APP.
AANWIJZINGEN

TYPE
11980/64

NR. Z2
DAT. 15-5-1956





VERVANGT
REMPLACE
ERSETZT
SUPERSEDES
NR.
DAT.

(M4) Meter indicating the emission-current.
(R601) Control for adjusting the filament current of the microscope tube.
(R606) Knob for adjusting the current through both focusing coils.
(R607) Knob for adjusting the total current through the focusing coils.
(R612) Knobs of the deflection system, for aligning the
(R613) electron-beam.
(R617) Knob for adjusting the condenser lens current.
(R623) Fine regulating knob for adjusting the magnification.
(R631) Knob for adjusting the zero point of the magnification meter. This can be found under the meter-panel on the left-hand side.
(R640) Regulating knob for focusing the image (objective lens).
(R641) Knob for extra fine adjustment of the objective lens current.
(W1) High tension switch.
(W2) Knob for coarse regulation of the magnification. The following magnification ranges can be selected with the aid of this knob.

a. When the objective lens is equipped with the pole shoes for a high resolving power (bore 1.8 mm. dia.)

Position W2	Lens switched on	Magnification on large screen
1 (extreme position to the right)	diffraction lens	) 0 - 8750X
2	diffraction lens	)
4.	intermediate lens	8000X - 90.000X

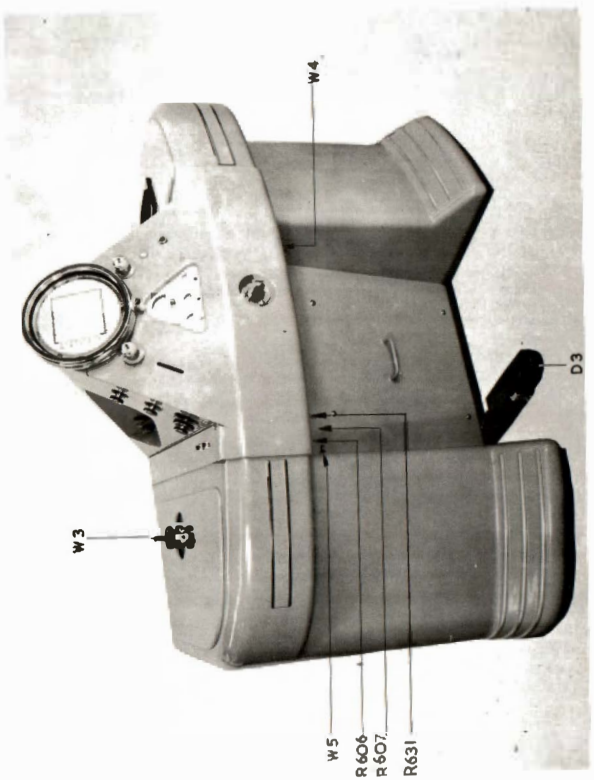
In position 3 only the objective lens and the projector lens determine the magnification. The intermediate lens and the diffraction lens are switched off. This position is particularly used for aligning the tube. The meter (M3) then indicates no magnification.

b. When the apparatus is suitable for diffraction. (In that case the objective lens is equipped with pole shoes with a large bore, i.e. 5 mm. dia.)

PHILIPS
EINDHOVEN
SERV. MED. APP.
AANWIJZINGEN

TYPE
11980/64

NR. Z 1
DAT. 15-5-1956

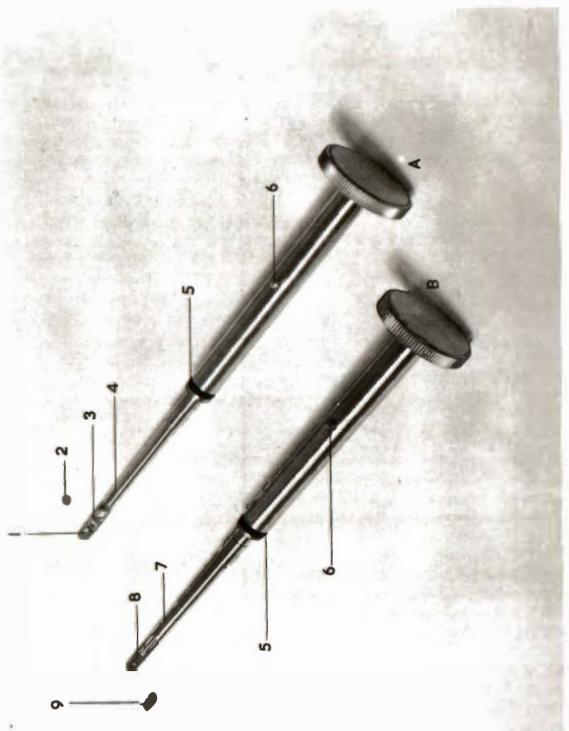


VERVANGT NR.
REMPLACE
ERSETZT DAT.

PHILIPS
EINDHOVEN
SERV. MED. APP.
AANWIJZINGEN

TYPE
11980/64

NR. Z 18
DAT. 15-5-1956



VERVANGT NR.
REMPLACE
ERSETZT DAT.
SUPERSEDES

Position W2	Lens switched on	Magnification
1	diffraction lens	)
2	diffraction lens	) 0 - 3500X)
4	intermediate lens	3400X - 35.000X

See point a for position 3.

(W3) Control of the valve box.
(W4) Switch for switching on the illumination of the meter panel.
(W6,Z32) Switch by means of which the high tension can be adjusted to half value. This switch is only used for aligning the tube (in combination with R654).

X.

AN APPLICATION TO RESEARCH

I should like to present a very brief summary of my recent work on the red pulp of the mammalian spleen. This work was done in partial fulfillment of an M.S. degree in Anatomy and publication in the not too distant future is anticipated. The reason for including this here is to illustrate the usefulness of the electron microscope in selected problems.

The argument concerning the "open" and "closed" intermediary circulation of the spleen has progressed through varying periods since it was first studied by Malphigi in 1686. (Bjorkman, 1947) The more recent important contributions have been by: Knisley (1936), MacKenzie (1941), Snook (1944, 1950 and 1958), Bjorkman (1947), Peck & Hoerr (1951 and Weiss (1957-59-61).

Knisley (1936), with a transillumination technique, proposed a closed circulation. MacKenzie (1941) using the same technique proposed an open circulation. Snook (1950) suggested the difference of opinion among these two was because Knisley had used rats and MacKenzie had used mice. Bjorkman (1947) has given an excellent historical review. Peck & Hoerr (1951) agreed with Knisley's original findings of a closed circulation.

Weiss (1957) has done the only previous electron microscopic studies of this organ. He proposed that the previously defined two types of channels, i.e. pulp cords and venous sinuses, were all equi-

valent vascular spaces. He demonstrated rather vaguely in this first paper, that the red pulp was an area consisting of the following structure: Reticulo-endothelial cells, basement membrane, reticulo-endothelial cells, sinal lumen, R-E cells, basement membrane, R-E cells, sinal lumen, etc. He proposed that the "cord spaces" appeared as such because they were flattened sinuses with the R-E cells pushed against one another. Since that time he published another article (1959) with excellent electron micrographs demonstrating these findings.

In my experiments I used the spleens of 3 rats and 4 rabbits. They were fixed in the modified Zetterquist-Caulfield formula decribed earlier. They were embedded in a 15:85 Methyl:Butyl methacrylate mixture with Luperco as a catalyst. They were incubated in a 60° C oven overnight and allowed to set two days before sectioning on the Porter-Blum microtome with glass knives. They were placed on Philips specimen carriers and some were stained with potassium permanganate, while others were not. They were viewed with the Philips EM 100B electron microscope. The micrographs were taken with the 35 mm. camera.

The observations I made were very similar to those of Weiss. The photograph in figure 18 is printed at low magnification and is an excellent example of multiple vascular channels. The basement membranes are clearly visible as are the reticular (R-E) cells lining both sides. It can be seen by the most casual observer that there are areas (A & B) which appear to be closed, and yet, following these same channels farther, are clearly open to blood flow. The cells lining the basement

membranes in these channels are identical to those in the neighboring channels.

Area (C) is a terminal arterial capillary ending upon and directly communicating with these channels. The basement membrane in these areas were shown in thicker silver-stained sections viewed by light microscopy to be the argyrophilic reticulum fibers. (Multiple areas in the red pulp showed this pattern previously described by Weiss). It is to be emphasized, however, that different areas showed different patterns. Many were subtle changes of the same configuration with the sinuses more completely collapsed as seen in the photographs in figures 19 & 20.

The nature of the sinal wall not described by Weiss is illustrated in this last series of photographs. Figure 21 shows an area of red pulp in which but a single basement membrane and single layer of reticular (R-E) cells line a sinus wall. This was often found throughout the red pulp. Figure 22 shows the junction of 2 reticular (R-E) cells separating vascular channels. The last set of photographs is three views (Figures 23, 24, 25) which picture a terminal arterial capillary ending in the red pulp. The ending assumes a rectangular configuration as it passes into the vascular channels of the red pulp. Some of its wall has a basement membrane with a layer of cytoplasm on each side, while another part has only a layer of cytoplasm between it and adjoining red cells. (See legend for further explanation).

After studying many areas of the red pulp of the spleen, I have found a similar pattern with many diverse subtleties. Not viewed here

are areas where macrophages appear to be on the verge of releasing themselves from a basement membrane. In view of all this, I have the following opinions:

(1) Weiss was quite accurate in his original description of many of the red pulp vascular channels.

(2) However, there is a diversity of subtle changes in the basic structure in various areas of red pulp. For example, often one layer of cytoplasm, rather than two, plus a basement membrane, separate vascular channels from one another.

(3) Macrophages are seen, which appear to be arising as mobilized reticular (R-E) cells.

The terms "closed" and "open" are archaic and their historical definitions are too restrictive to be applied to the red pulp of the spleen. All vascular spaces beyond true capillaries are lined by reticular (R-E) cells, usually accompanied by a basement membrane (reticulum fiber). There is great anatomical variation in the course of the blood through these spaces. This accounts for the diverse findings of the various investigators prior to electron microscopic studies.

Plates

Chapter X.

Plates

Chapter X.

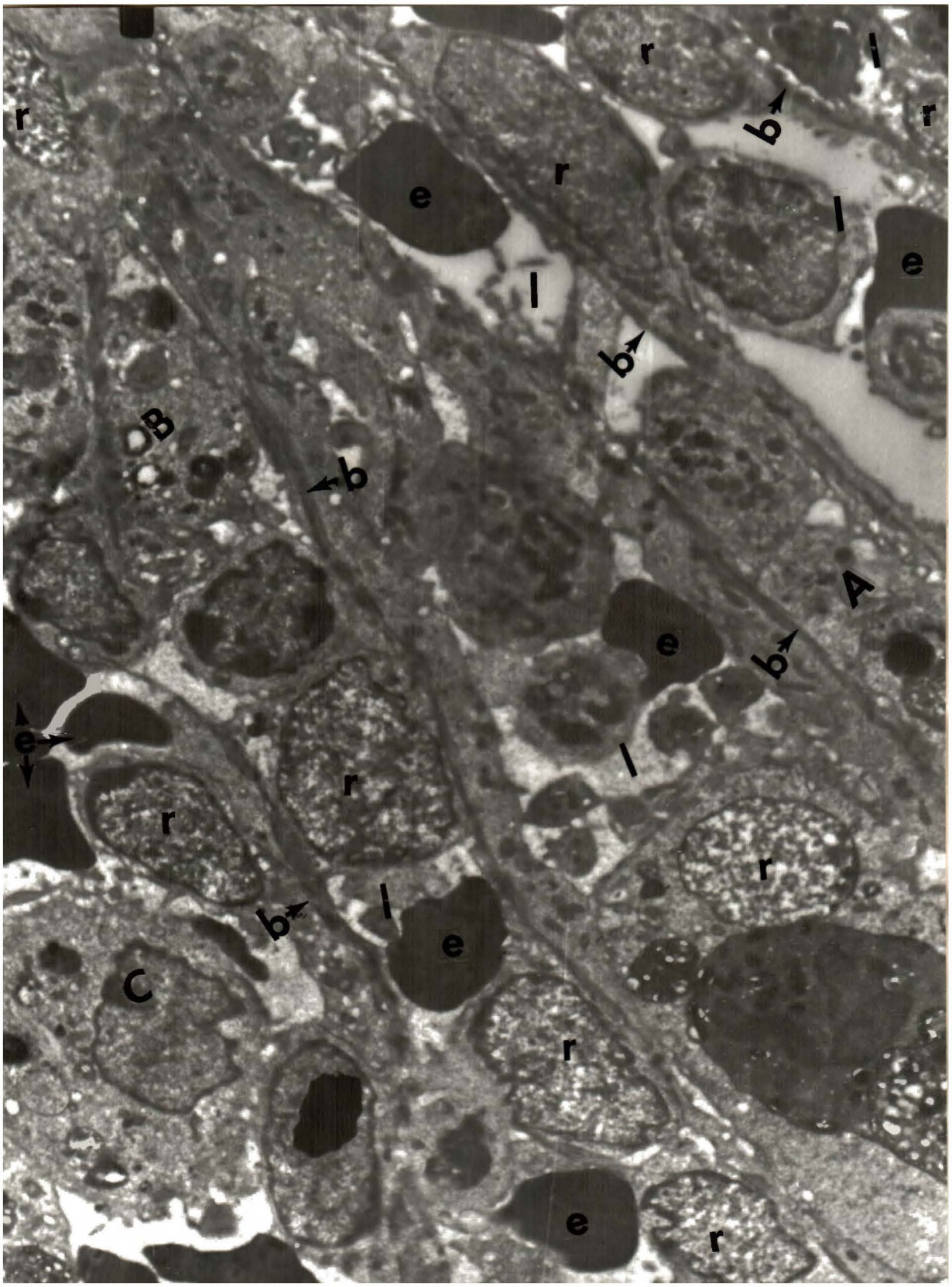

r
r
l
b
r
e
r
l
l
e
b
B
b
A
e
b
e
l
r
r
r
b
l
e
C
r
e
r

Plate XIV.

Figure 19. Collapsed Vascular Channels in the Red Pulp of the Spleen. (5350X, 60KV).

(b) Basement membrane (Argyrophilic reticulum fibers).

(e) Erythrocyte within a vascular channel.

(l) Vascular channel (sinal lumen).

(r) Reticular (Reticulo-endothelial) cells.

Note the similarity of the cells lining the basement membrane. The sequence of structures from one vascular channel is lumen (l), reticular cell (r), basement membrane (b), reticular cell (r), lumen (l), reticular cell (r), etc.

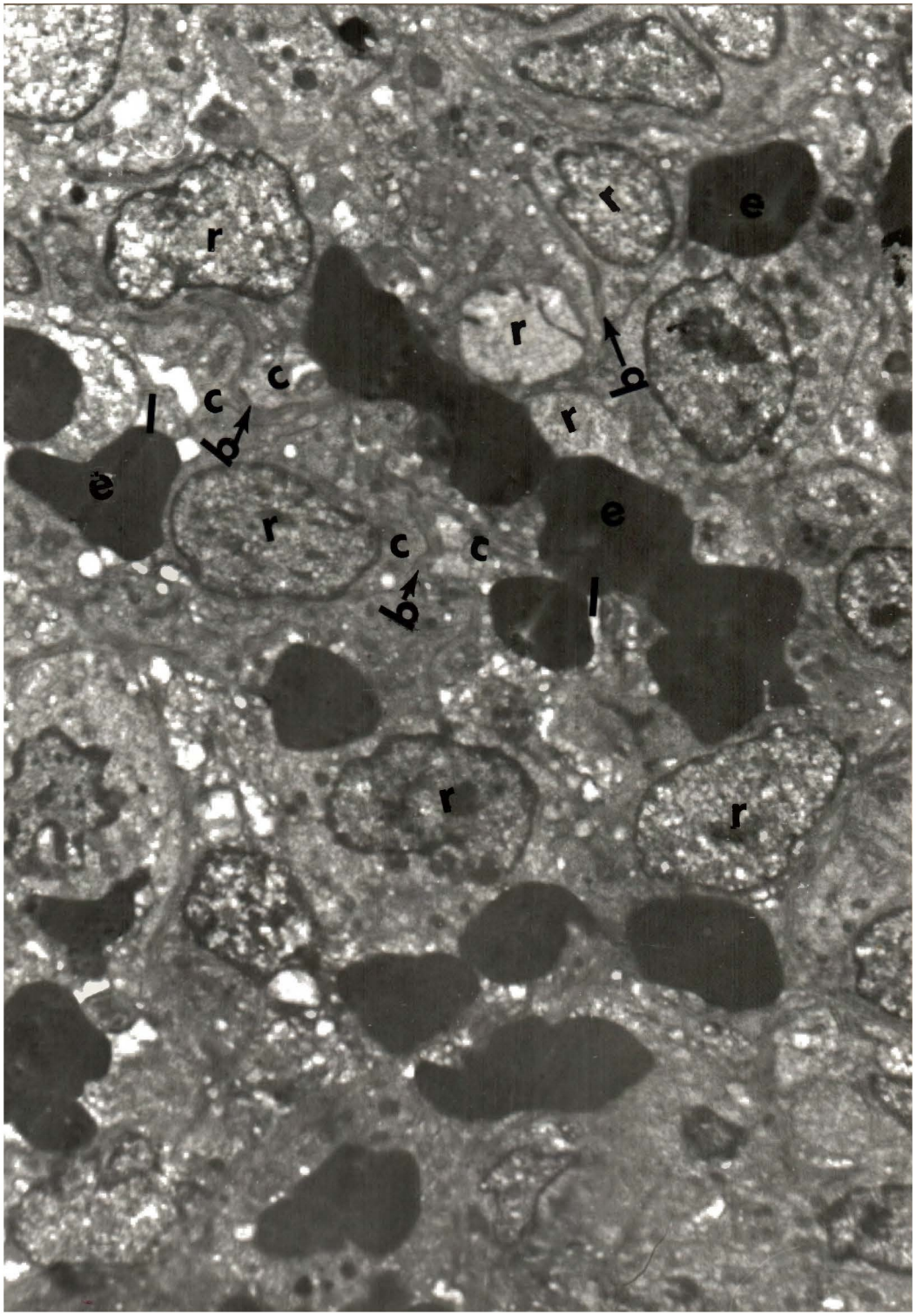

r
r
e
r
b
c
c
b
l
e
r
c
c
b
e
l
r
r

Plate XV.

Figure 20. Higher Magnification of the Collapsed Vascular Channels seen in Figure 19. (10250X, 60KV).

(b) Basement membrane (Argyrophilic reticulum fibers).

(e) Erythrocyte within a vascular channel (l).

(l) Vascular channel (sinal lumen).

(r) Reticular (Reticulo-endothelial) cells, and their cytoplasm (c).

Note same configuration as Figure 19.

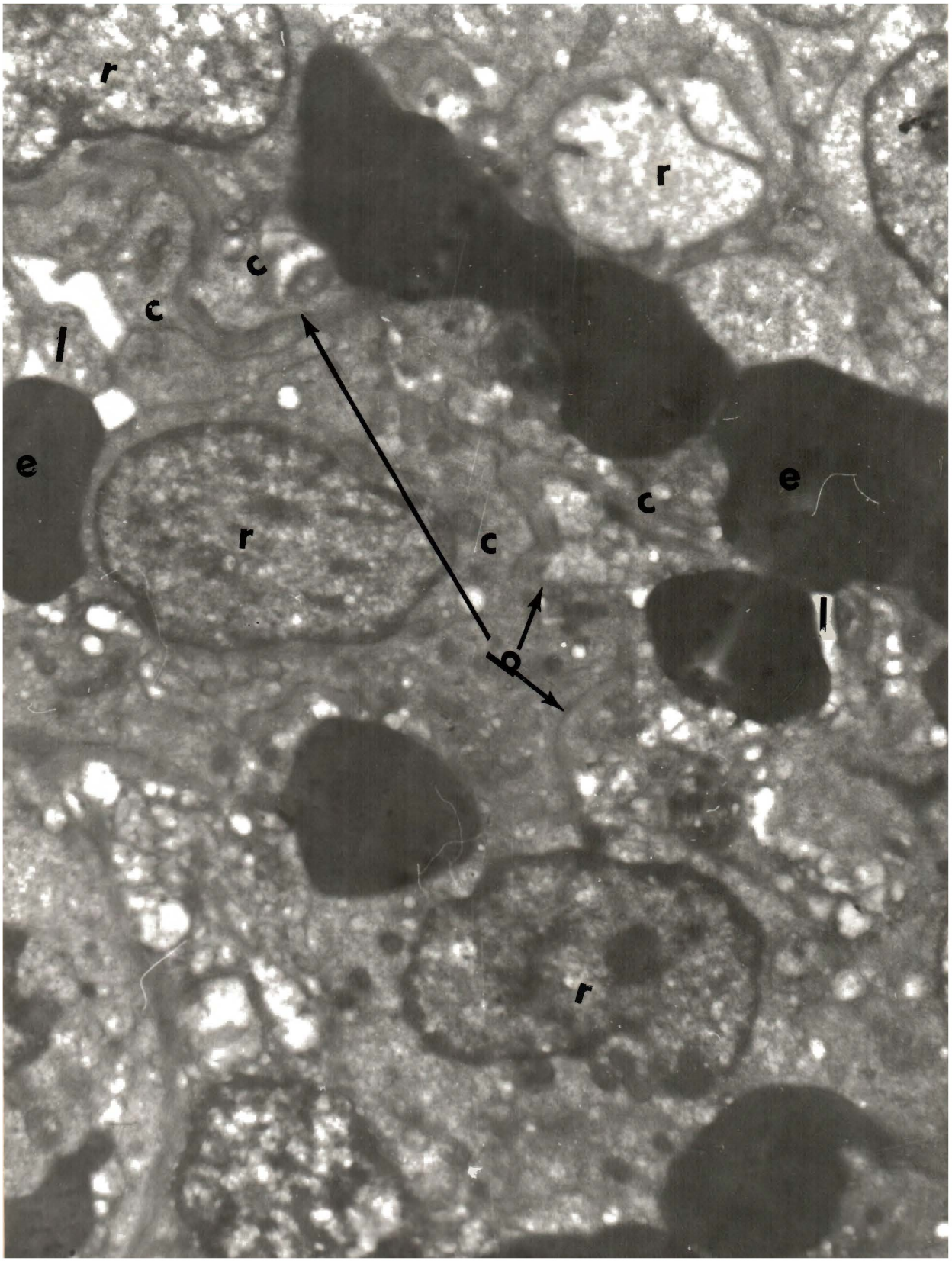

r
r
c
c
l
e
r
c
c
e
l
p
r

Plate XVI.

Figure 21. Vascular Channels Separated by a Basement Membrane and a Single Layer of Reticular Cells. (10250X, 60KV).

(b) Basement membrane.

(e) Erythrocyte within vascular channel (l).

(l) Vascular channel (sinal lumen).

(r) Reticular (reticulo-endothelial) cells and their cytoplasm(c).

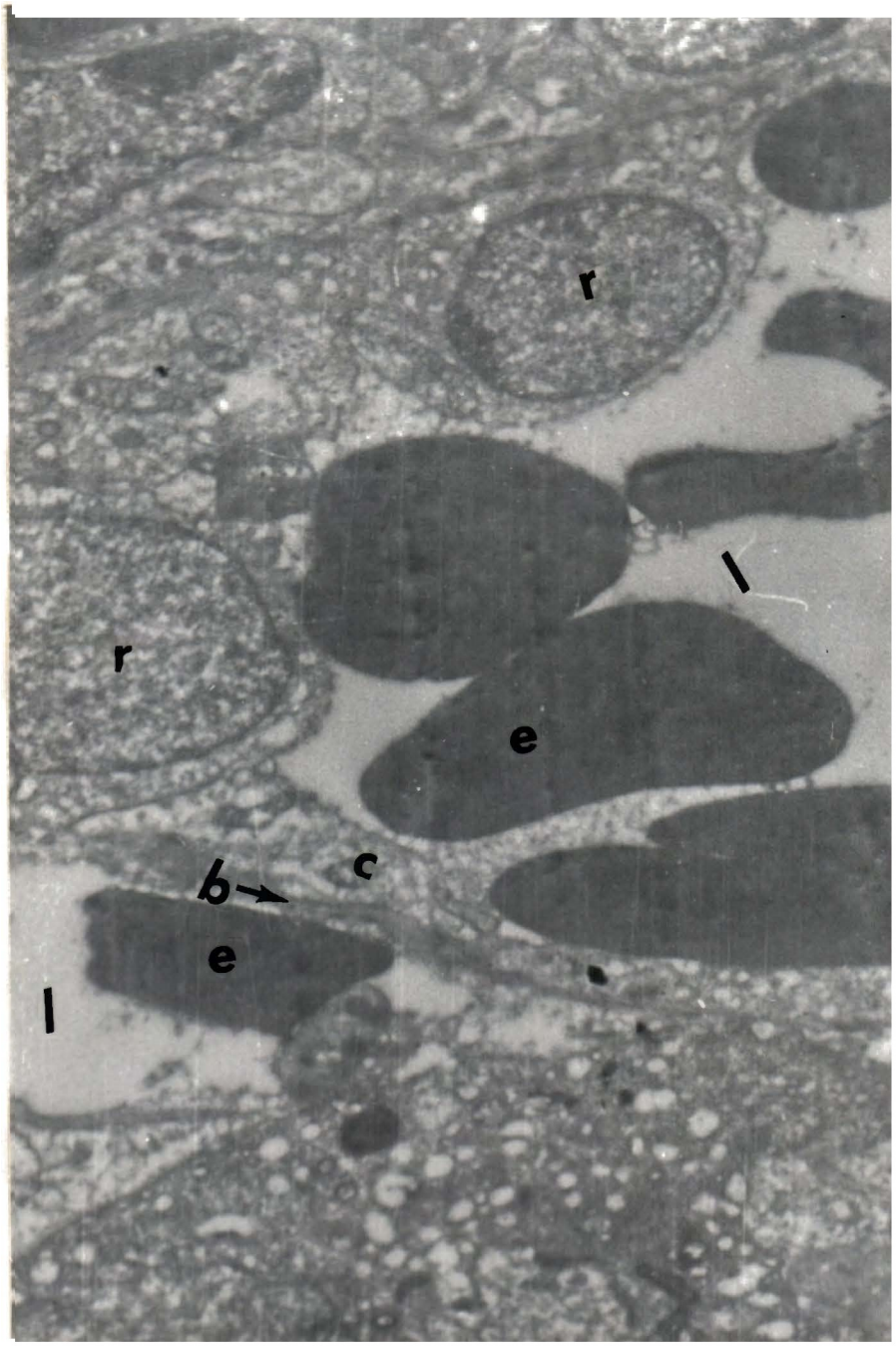

r
l
r
e
b
c
e
l

Plate XVII.

Figure 22. Vascular Channels Separated by a Single Layer of Cytoplasm. (15050X, 60KV).

(b) Basement membrane ?

(e) Erythrocyte in vascular channels (l).

(l) Vascular channel (sinal lumen).

(r) Reticular (reticulo-endothelial) cells and their cytoplasm (c).

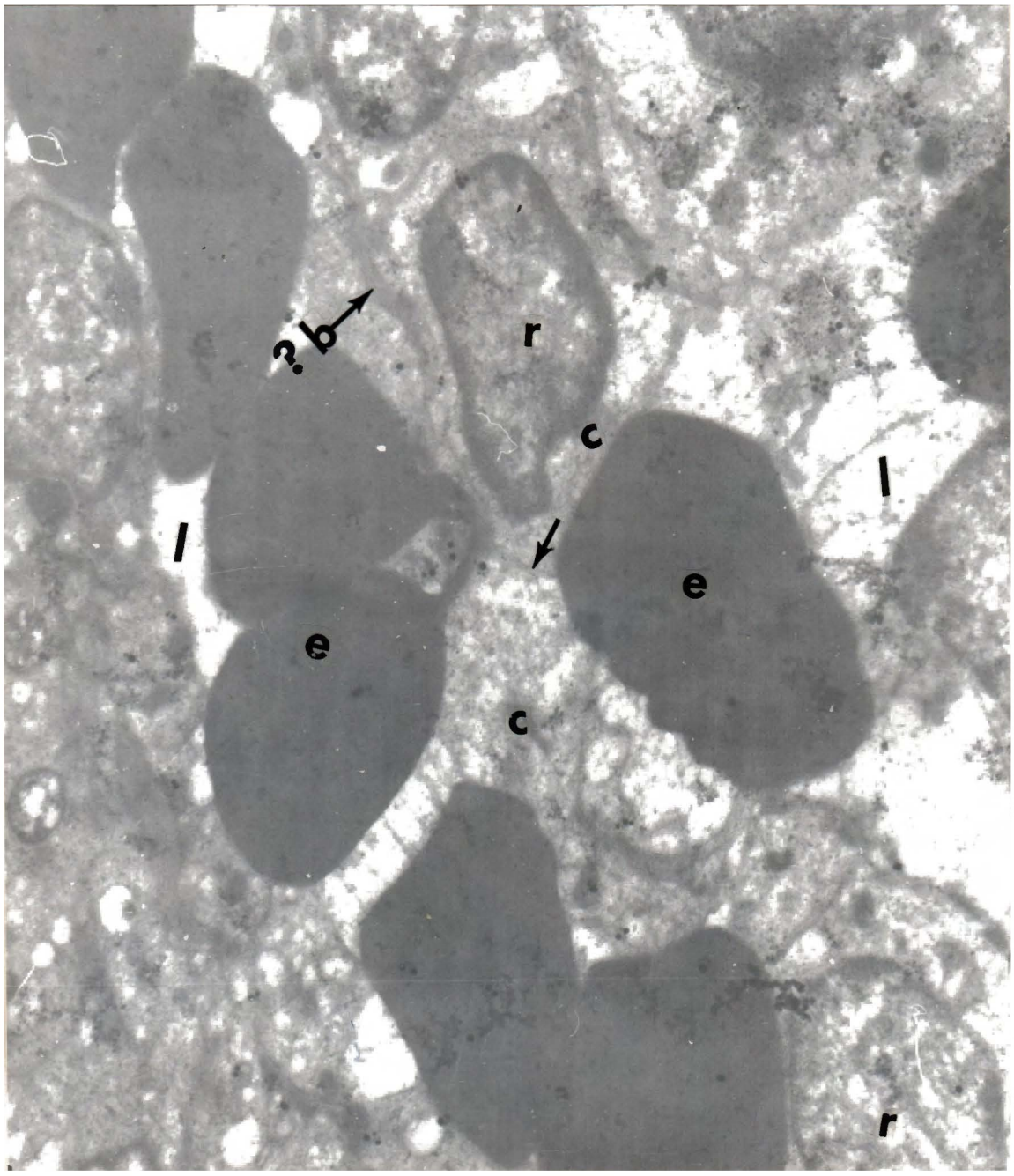

?b
r
c
l
l
e
e
c
r

Plate XVIII.

Figure 23. Terminal Arterial Capillary Communicating Directly with the Vascular Channels of the Red Pulp. (5350X, 80KV).

(a) Lumen of terminal arterial capillary.

(b) Basement membrane.

(e) Erythrocyte in vascular channel (L).

(l) Vascular channel (sinal lumen).

(r) Reticular (reticulo-endothelial) cell.

(T) Lumen of rectangular space believed to area of transition from terminal arterial capillary to vascular channels of red pulp.
As one traces the boundaries of space he notes that it is bounded by 3 types of walls: A part with the (r),(bm) and (r) configuration; a part with (r),(bm) configuration and a part with only cytoplasm of one reticular cell(r) separating this space from the surrounding vascular channels. Figures 24 and 25 show this at higher magnification.

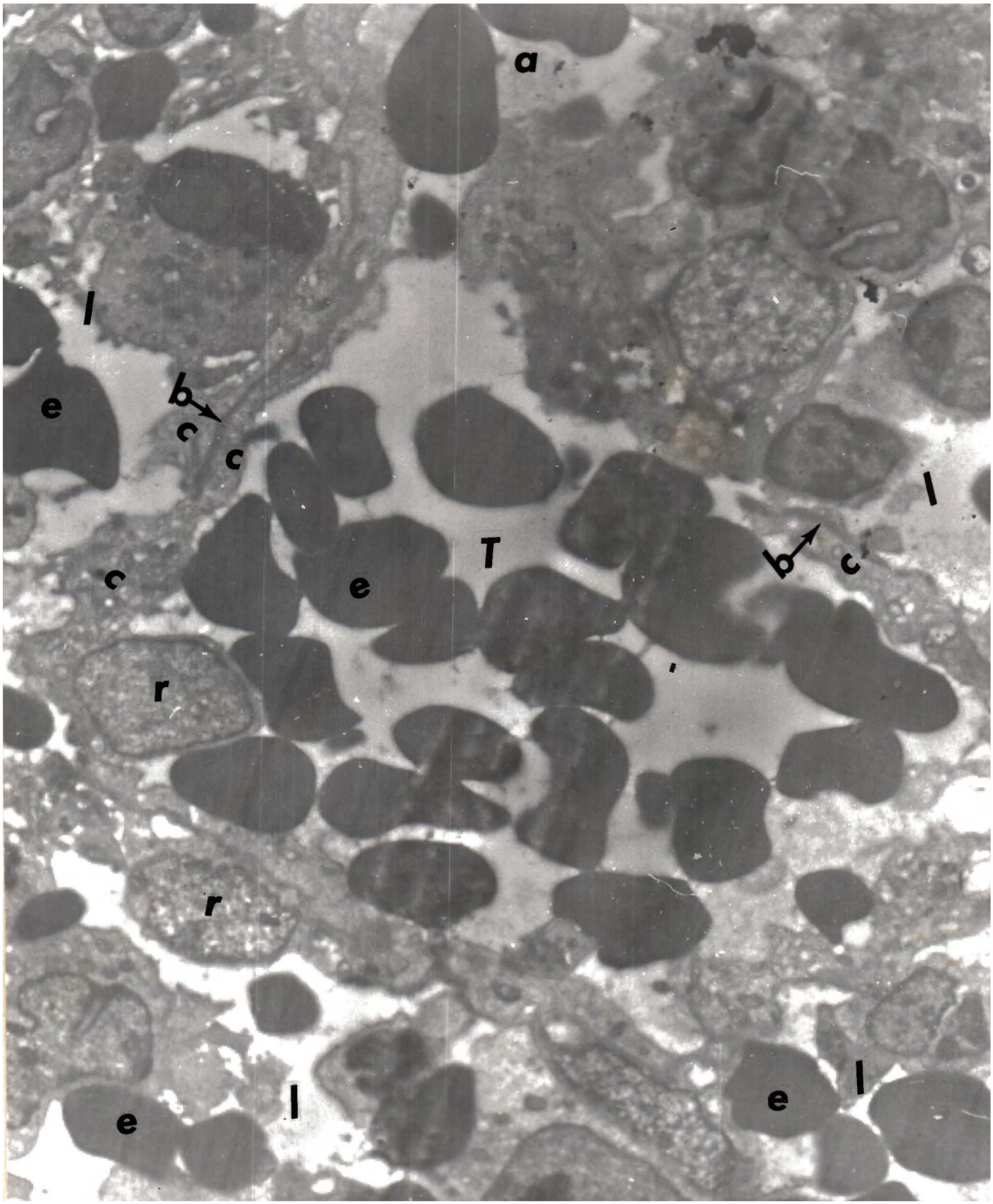

a
l
e
b
c
c
l
T
b
c
c
e
r
r
e
l
e
l

Plate XIX.

Figure 24. Higher Magnification of the Wall of the Transitional Lumen in Figure 23. (10, 250X, 80KV).

(b) Basement membrane.

(e) Erythrocyte in vascular channel (l).

(l) Vascular channel (sinal lumen).

(r) Reticular (reticuloendothelial) cell with cytoplasm (c).

(T) Lumen of rectangular space believed to be area of transition from terminal arterial capillary to vascular channels of red pulp.

Note area of wall composed only of cytoplasm (c) of reticular (r) cell. This is separated from area with basement membrane and two layers of cytoplasm by unmarked arrow.

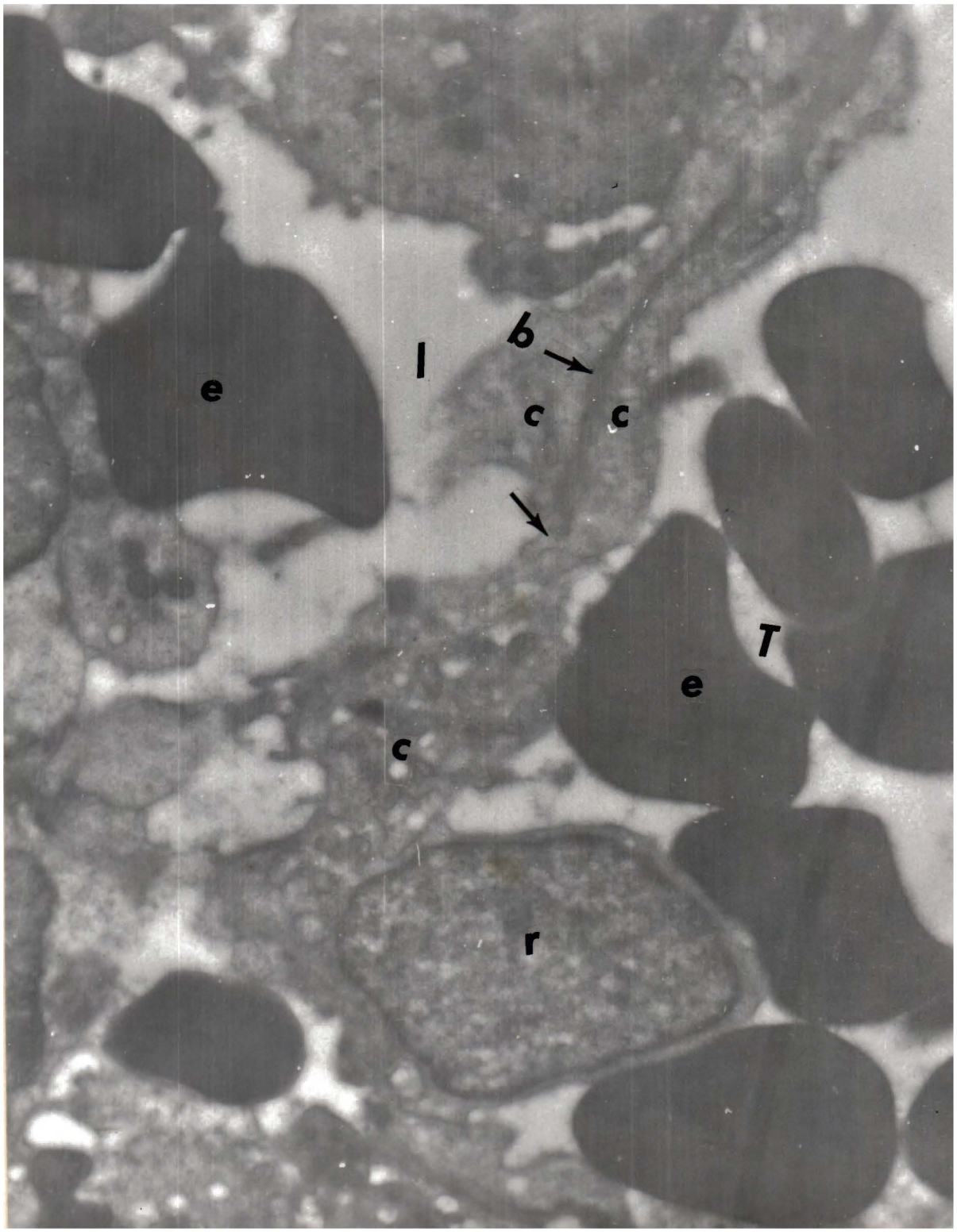

e
l
b
c
c
T
e
c
r

Plate XX.

Figure 25. Higher Magnification of the Wall of the Transitional Lumen in Figure 23.

(b) Basement membrane.

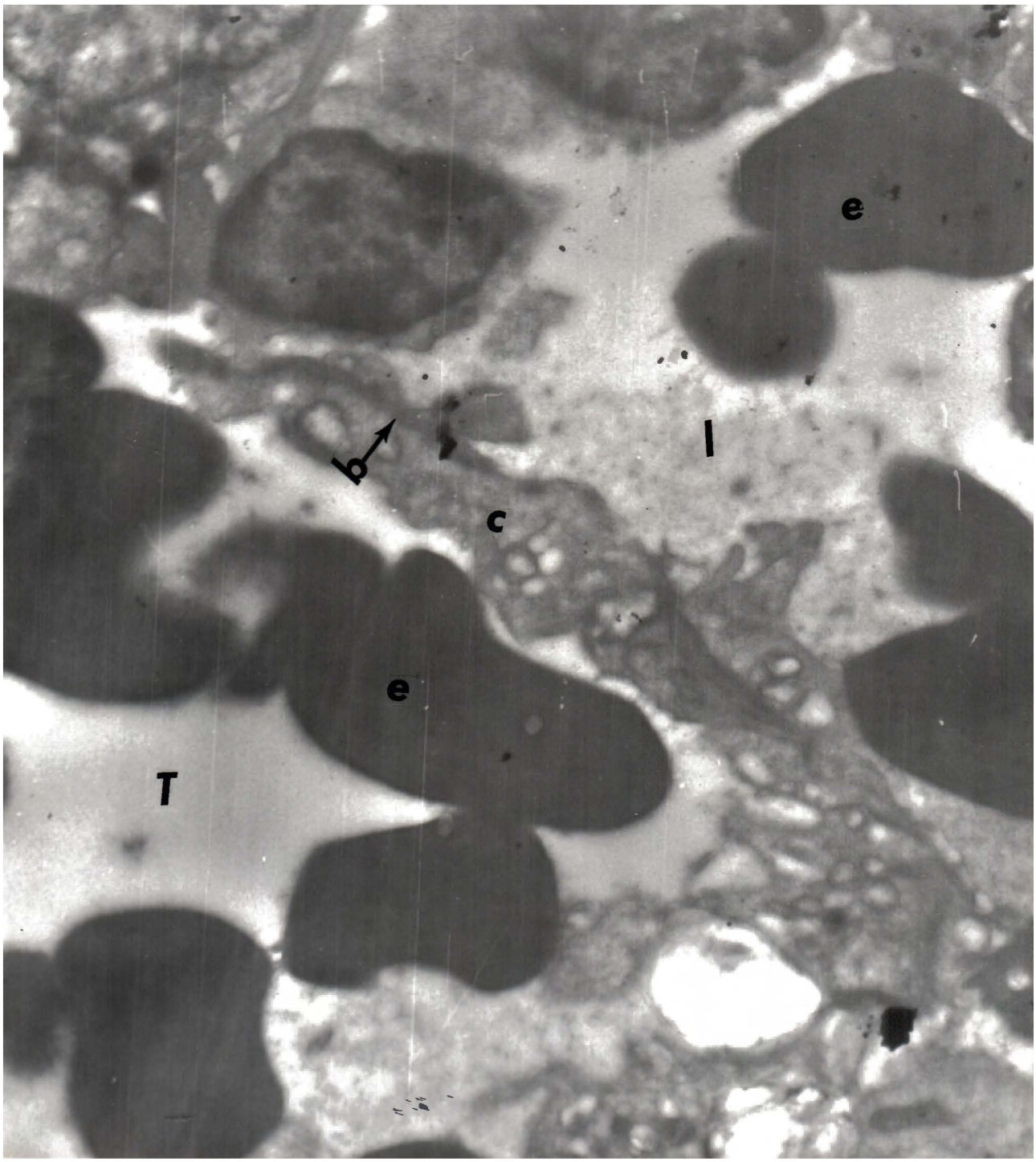
(e) Erythrocyte in vascular channel.

(l) Vascular channel (sinal lumen).

(r) Reticular (reticulo-endothelial) cell with cytoplasm (c).

(T) Lumen of rectangular space believed to be area of transition from terminal arterial capillary to vascular channels of red pulp.

Note one layer of cytoplasm (c) with a basement membrane (b).


e
b
l
c
e
T

XI.

SUMMARY AND CONCLUSION

The electron microscope has brought insight into a previously unexplored macromolecular universe. With its amazing capabilities have come many limitations. It is important that all those associated be educated concerning both these aspects. The author believes it adaptable to essentially all fields of biological research. Cooperation and correlation by all concerned are necessary. The first chapter deals primarily with these aspects of electron microscopy.

In chapters two through six, the many processes involved in preparing specimens for use in the electron microscope are presented. These include prefixation specimen preparation, fixation, embedding, sectioning, staining, and mounting of the tissue specimen. These have been so presented to provide the student with a basic understanding of the fundamental processes involved. In this way, he can utilize his individual innate ability to originate and modify procedures to meet his various needs. It is hoped that with this ability he can stimulate the further growth and development of electron microscopy.

Chapters seven through nine concern themselves with the electron microscope itself. A brief history of the development of microscopy is presented in chapter seven. In chapter eight I have attempted to present an insight into the physical principles involved in the construction and the operation of the electron microscope. This builds

a foundation for the proper operation of the electron microscope as described in "cook book" style in chapter nine. The manipulation of the electron microscope should soon become a part of his routine daily abilities. It is hoped that the diagrams and photographs placed in these chapters will make the learning process easier.

Interpretation of electron micrographs is not discussed. The student is urged to read chapter one of Maximow & Bloom's Textbook of Histology for a basic understanding of ultrastructural detail before consulting the numerous good articles available in the current literature.

The electron microscope has given us a magnificent tool with which to explore the ultrastructure of living matter. Its application in all fields of medical research (clinical and basic science) is rapidly being recognized. It is hoped that the presented material will be of assistance in the development of the future electron microscopist, and, in so doing, further the advancement of medical science itself.

BIBLIOGRAPHY

Literature Cited

Bahr, G. F., Osmium Tetroxide and Ruthenium Tetroxide, Exp. Cell Res. 7:457-479, 1954.

Birbeck, M. S. C., and Mercer, E. H., Some Applications of an Epoxide Embedding Medium, Demonstration at 15th Meeting of Electron Microscope Society of America, Boston, 1957.

Bjorkman, F. E., The Splenic Circulation, Acta Med. Scand. Suppl. 191:1-89, 1947.

Borysko, Emil, Methacrylate Embedding I. A Study of the Polymerization Damage Phenomenon by Phase Contrast Microscopy, J. Biophys. Biochem. Cytol. 2:3-14, Suppl., 1956.

_____, Methacrylate Embedding II. Methods for the Sectioning of Optically Selected Single Cells, the Orientation of the Plane of Sectioning and the Identification of the Region of the Specimen Included in the Sections, J. Biophys. Biochem. Cytol. 2:15-20, Suppl., 1956.

Cameron, D. A., A Note on Breaking Glass Knives, J. Biophys. Biochem. Cytol. 2:57, Suppl., 1956.

Caulfield, B., Effects of Varying the Vehicle for OsO_4 in Tissue Fixation, J. Biophys. Biochem. Cytol. 3:827-830, 1957.

Claude, Albert, and Fullman, E. F., The Preparation of Sections of Guinea Pig Liver for Electron Microscopy, J. Exp. Med. 83:499, 1946.

Clevenger, M. R., and Low, F. N., Some Experiments with Polyesters as Embedding Media for Electron Microscopy, Demonstration at Amer. Assoc. Anat. Meetings, Chicago, 1961.

Dalton, A. J., A Chrome-Osmium Fixative for Electron Microscopy, Anat. Rec. 121:281, 1955.

_____, et al., J. Natl. Cancer Inst. 11:439, 1950. Cited by: Pease, D. C., Histological Techniques for Electron Microscopy, New York & London, Academic Press, 1960, p. 31.

_____, and Zeigel, R. F., A Simplified Method of Staining Thin Section of Biological Material with PbOH for Electron Microscope, J. Biophys. Biochem. Cytol. 6:409, 1960.

Fernandez-Moran, Humberto, Electron Microscopy of Retinal Rods in Relation to Localization of Rhodopsin, Science 129:12 85, 1959.

Gavin, M. A., and Lloyd, B. J. Jr., Knives of High Silicate Content Glass for Thin Sectioning, J. Biophys. Biochem. Cytol. 5:507-510, 1959.

Glauert, M., and Glauert, R. H., Araldite as an Embedding Medium for Electron Microscopy, J. Biophys. Biochem. Cytol. 4:191, 1958.

_____, et al., A New Embedding Medium for Electron Microscopy, New York, Toronto & London, McGraw-Hill Book Co., Inc., 1953, p. 211.

Hall, C. E., Introduction to Electron Microscopy, New York, Toronto & London, McGraw-Hill Book Co., Inc., 1953, p. 211.

_____, et al., J. Appl. Physics 16:459, 1945. Cited by : Pease, D. C., Histological Techniques for Electron Microscopy, New York & London, Academic Press, 1960, p. 148.

Houch, E., and Dempsey, E. W., Cytological Staining Procedures Applicable to Methacrylate-Embedded Tissues, Stain Tech. 29:207-211, 1954.

Hungerford, D. A. and Di Berardino, Marie, Cytological Effects of Prefixation Treatment, J. Biophys. Biochem. Cytol. 4:391, 1958.

Huxley, H. E., Some Observations on the Use of Araldite as an Embedding Medium, Paper at 15th Meeting of Electron Microscope Society of America, Boston, 1957.

Jansen, P. C., An Introduction to the Electron Microscope, Science and Industry, Vol. 7, NR.2:33-52, 1960.

Kellenberger, E., et al., L'utilisation d'un Copolymere du Groupe des Polyesters comme Materiel d'Inclusion en Ultra Microtomie, Experentia 12:421, 1956.

Knisley, M. H., Microscopic Observation of the Circulatory System of Living Unstimulated Mammalian Spleens, Anat. Rec. 65: 23-50, 1936.

Knisley, M. H., Microscopic Observation of the Circulatory System of Living Traumatized Spleens and of Dying Spleens, Anat. Rec. 65:131-148, 1936.

Knoll, M., and Ruska, E., The Electron Microscope, Z. Physik. 78:318-319, 1932. Cited by: Hall, C. E., Introduction to Electron Microscopy, New York, Toronto & London, McGraw-Hill Book Company, Inc., 1953, Chap. 8.

Latta, J. S., Personal communication to the author, 1961.

Lawn, A. M., The Use of Potassium Permanganate as an Electron Dense Stain for Sections of Tissue Embedded in Epoxy Resins, J. Biophys. Biochem. Cytol. 6:197, 1960.

Le Poole, J. B., A New Electron Microscope with Continuously Variable Magnification, Philips Tech. Rev. 9:33-45, 1947.

Maaløe, O., and Birch-Anderson, A., On the Organization of the Nuclear Material in Salmonella Typhimurium, 6th Symposium of the Society for General Microbiology, Bacterial Anatomy, Cambridge, The University Press, 1956, p. 261. Cited by: Glauert, M., and Glauert, R. H., Araldite as an Embedding Medium for Electron Microscopy, J. Biophys. Biochem. Cytol. 4:191, 1958.

MacKenzie, David Jr., et al., Studies on the Microscopic Anatomy and Physiology of Living Transilluminated Mammalian Spleens, Amer. J. Anat. 68:397-453, 1941.

Maximow, A. A., and Bloom, William, Textbook of Histology, 7th Ed., Philadelphia, W. B. Saunders Co., 1957, Chap. 1.

Moore, D. H., and Grimly, P. M., Problems in Methacrylate Embedding for Electron Microscopy, J. Biophys. Biochem. Cytol. 3:255, 1957.

_____, et al., Some Effects of the Microtome Knife and Electron Beam on Methacrylate-Embedded Thin Sections, J. Biophys. Biochem. Cytol. 2:21-28, Suppl., 1956.

Newman, J. B., et al., Ultramicrotomy by a New Method, J. Res. Natl. Bureau Standards 43:183, 1949.

Operation Manual for Electron Microscope, Philips Model 100B, Philips Electronics, 1956, p. C6.

Operation Manual for Porter-Blum Microtome, Ivan Sorvall, Inc., Norwalk, Conn.

Ornstein, Leonard, How Thin Should a Section Be?, J. Biophys. Biochem. Cytol. 2:53-55, Suppl., 1956.

Palade, G. E., A Study of Fixation for Electron Microscopy, J. Exp. Med. 95:285, 1952.

_____, Proc. Third International Conference Electron Microscopy, London, 1954, p. 129, Royal Micro. Soc. London, 1956. Cited by: Pease, D. C., Histological Techniques for Electron Microscopy, New York & London, Academic Press, 1960, p. 60.

Pallie, W., and Pease, D. C., Prefixation Use of Hyaluronidase to Improve in situ Preservation for Electron Microscopy, J. Ultrastructural Res. 2:1-7, 1958.

Peachey, L. D., A Study of Section Thickness and Physical Distortion Produced during Microtomy, J. Biophys. Biochem. Cytol. 4:233, 1958.

_____, and Satir, G., A Simple Method for Reducing Compression Artifacts, J. Biophys. Biochem. Cytol. 4:345, 1958.

Pease, D. C., Histological Techniques for Electron Microscopy, New York & London, Academic Press, 1960, pp. 14, 23, 46, 39-42, 77, 111, 110-112, 125-129, 135.

_____, and Baker, R. F., Sectioning Techniques for Electron Microscopy, Proc. Soc. Exp. Biol. and Med. 67:470-474, 1948.

_____, Electron Microscopy of the Kidney, Amer. J. Anat. 87:349, 1950.

Peck, M., and Hoerr, L., The Intermediary Circulation in the Red Pulp of the Mouse Spleen, Anat. Rec. 109:447-478, 1951.

Porter, K. R., Anat. Rec. 106:145, 1950. Cited by: Pease, D. C., Histological Techniques for Electron Microscopy, New York & London, Academic Press, 1960, p. 31.

_____, and Blum, J., A Study in Microtomy for the Electron Microscope, Anat. Rec. 117:685-712, 1953.

_____, and Kallman, F., The Properties and Effects of Osmium Tetroxide as a Tissue Fixative with Special Reference to Its Use for Electron Microscopy, Exp. Cell Res. 4:127-141, 1 953.

Ryter, Antoinette, and Kellenberger, E., L'inclusion au Polyester pour L'ultramicrotomie, J. Ultrastructural Res. 2:200, 1958.

Sjostrand, F. S., and Baker, R. F., J. Ultrastructure Res. 1:239, 1958. Cited by: Pease, D. C., Histological Techniques for Electron Microscopy, New York & London, Academic Press, 1960, pp. 32-60.

Smith, Jerome, Personal communication to the author, 1960.

Snook, Theodore, The Histology of Vascular Terminations in the Rabbit's Spleen, Anat. Rec. 130:711, 1958.

_____, A Comparative Study of the Vascular Arrangements in Mammalian Spleens, Amer. J. Anat. 87:31-78, 1950.

Tokuyasu, K., and Okamura, S., A New Method for Making Glass Knives for Thin Sectioning, J. Biophys. Biochem. Cytol. 5:305, 1959.

Van Breeman, V. L., Personal communication to the author, 1960.

Ward, R. T., J. Appl. Physics, 1959. Cited by: Pease, D. C., Histological Techniques for Electron Microscopy, New York & London, Academic Press, 1960, p. 75.

Watson, M. L., Staining of Tissue Sections for Electron Microscopy with Heavy Metals, J. Biophys. Biochem. Cytol. 4:475, 1958.

Weiner, Stanley, A New Method of Glass Knife Preparation for Thin Section Microscopy, J. Biophys. Biochem. Cytol. 5:175-177, 1959.

Weiss, L. P., A Study of the Structure of Splenic Sinuses in the Albino Rat with the Light Microscope and the Electron Microscope, J. Biophys. Biochem. Cytol. 3:599-611, 1957.

_____, An Experimental Study of the Organization of the Reticulo-Endothelial System in the Red Pulp of the Spleen, J. Anat. (London) 93:4, 1959.

_____, Observations on the Red Pulp of the Spleen of Rabbits and Dogs by Electron Microscopy, presented at Amer. Assoc. Anat., Chicago, 1961.

_____, Personal communication to the author, 1961.

Williams, R. C., and Kallman, F., Interpretations of Electron Microscopy of Single and Serial Sections, J. Biophys. Biochem. Cytol. 1:301, 1955.

Zetterquist, H., The Ultra Structural Organization of the Columnar Absorbing Cells of the Mouse Jejunum, Dept. Anat. Karolinska Institute, Stockholm, 1956.

Zinsser, Hans, et al., Zinsser Microbiology, New York, Appelton-Century-Crofts, Inc., 1960, p. 2.

General References

Afzelius, Bjorne, Electron Microscopy of the Sperm Tail -- Results Obtained with a New Fixative, J. Biophys. Biochem. Cytol. 5:269-278, 1959.

Altschul, R., and Hummason, F. A., Minimal Vascular Injection of the Spleen, Anat. Rec. 97:259-264, 1947.

Baker, R. F., and Modern, F. W. S., Controlled Fixation with Osmium Tetroxide, Anat. Rec. 114:181-188, 1954.

Bencosme, S. A., et al., A Rapid Method of Localization of Tissue Structures or Lesions for Electronmicroscopy, J. Biophys. Biochem. Cytol. 5:508-509, 1959.

Bernstein, M. H., Iron as a Stain for Nucleic Acids in Electron Microscopy, J. Biophys. Biochem. Cytol. 2:633-634, 1956.

Coslett, V. E., Practical Electron Microscopy, New York, N.Y., Academic Press, Inc., 1951.

Dalton, A. J., A Chromic-Osmium Fixative for Electron Microscopy, Anat. Rec. 121:281, 1955.

Davies, M. R., and Wallace, R., The Use of Carbon Films in the Culture of Tissue Cells for Electron Microscopy, J. Biophys. Biochem. Cytol. 4:231, 1958.

Dempsey, E. W., The Use of Silver Nitrate as a Vital Stain and its Distribution in Several Mammalian Tissues as Studied with the Electron Microscope, J. Biophys. Biochem. Cytol. 1:111-118, 1955.

Epstein, M. A., A Diamond Knife Holder for Microtomes Designed to Take Glass Knives, J. Biophys. Biochem. Cytol. 4:539, 1958.

Feder, Ned, and Sidman, R. L., Methods and Principles of Fixation by Freeze Substitution, J. Biophys. Biochem. Cytol. 4:593, 1958.

Fleming, W. W., and Parpart, A. K., Structure of the Intermediate Circulation of the Rat Spleen, Angiology 10:28, 1959.

Freeman, J. A., and Low, F. N., Some Experiments with Chromium Compounds as Fixers for Electron Microscopy, J. Biophys. Biochem. Cytol. 2:629-632, 1956.

Goldberg, G. M., Lymphatics of the Spleen, J. Anat. (London) 92:310-14, 1958.

Harris, Patricia, and Mazia, Daniel, The Use of Mercuric Bromphenol Blue as a Stain for Electron Microscopy, J. Biophys. Biochem. Cytol. 5:343-347, 1959.

Harvere, Etienne, A New Technique for Carbon Films, J. Biophys. Biochem. Cytol. 4:133, 1958.

Howatson, A. F., and Almeida, J. D., A Method for the Study of Cultured Cells by Thin Sectioning and Electron Microscopy, J. Biophys. Biochem. Cytol. 4:115, 1958.

Latta, H., and Hartman, J. F., Use of a Glass Edge in Thin Sectioning for Electron Microscopy, Proc. Soc. Exp. Biol. Med. 74:436, 1950.

Lewis, O. T., The Development of the Circulation in the Spleen of the Foetal Rabbit, J. Anat. 90:282-289, 1956.

Luft, J. H., and Bennet, H. S., S-collidine as a Basis for Buffering Fixative, J. Biophys. Biochem. Cytol. 6:113, 1959.

Merriam, R. W., The Contribution of Lower Oxides of Osmium to the Density of Biological Specimens in Electron Microsco py, J. Biophys. Biochem. Cytol. 4:519, 1958.

Novikoff, A. B., Preservation of the Fine Structure of Isolated Liver Cell Particulates with Polyvinylpyrrollidone-sucrose, J. Biophys. Biochem. Cytol. 2:65-66, 1956.

Ohta, Y., Cubical Anatomy of Several Ducts and Vessels by Injection Method of Acrylic Resin, III. On the Vascular System of the Spleen in Some Animals, Okajimas Folio Anatomica Japonica (Tokyo) June, 1957, pp. 13-41.

Palay, S. L., Frontiers in Cytology, New Haven, Yale University Press, 1958.

Parpart, A. K., et al., The Microcirculation of the Spleen of the Mouse, Angiology 6:350-362, 1955.

Peachey, L. D., A Device for Staining Tissue Section for Electron Microscopy, J. Biophys. Biochem. Cytol. 5:511-513, 1959.

Porter, Keith, et al., A Study of Tissue Culture Cells by Electron Microscopy, J. Exp. Med. 81:233-246, 1945.

Watson, M. L., Carbon Films and Specimen Stability, J. Biophys. Biochem. Cytol. 2, Suppl., 1956.

_____, The Use of Carbon Films to Support Tissue Sections for Electron Microscopy, J. Biophys. Biochem. Cytol. 1:163, 1955.

_____, The Nuclear Envelope, J. Biophys. Biochem. Cytol. 1:257, 1955.

Wislocki, G. B., and Dempsey, E. W., Electron Microscopy of Human Placenta, Anat. Rec. 123:133, 1955.

_____, Electron Microscopy of the Placenta of a Rat, Anat. Rec. 123:33, 1955.