Electron Microscopy: Quantitative Techniques

The central role which microscopy has played in the development of classical biology and medicine is widely recognized. After a brief induction period in the 1940's when basic techniques were being worked out, the electron microscope assumed the same function in modern biological science that the light microscope served in the classical period. A large part of classical microscopy however is purely descriptive. With electron microscopy we have the same advantages of pictorial display but it appears that the investigator will need to take a more quantitative approach using physical and chemical methods of analysis. In order to review the present developmental state of quantitative techniques in electron microscopy, a symposium was held at the Walter Reed Medical Center in Washington, D.C., 30 March–3 April 1964. Of the 42 participants, about one-half were from the United States, 10 from West Germany, 6 from England, and 1 each from Canada, Holland, and Japan.

Image Formation

An evaluation of the meaning of electron micrographs requires an understanding of the nature of electron beams, the optical properties of the lenses and apertures traversed by the beam, and the interaction between beam and specimen structure which produces the modulations of intensity observed in the final image. Discussions of the optics of the lens systems were presented by L. Marton, O. Scherzer, and F. Lenz. D. Gabor discussed image formation from the point of view of information theory in which the optical tract of the electron microscope is regarded as a two-dimensional analogue of the communication channels familiar in electrical engineering. This led him to suggest methods of processing electron micrographs to obtain information not otherwise evident to the eye. Electron scattering by matter was dealt with in its various aspects in separate dissertations by H. Fowler, V. Cosslett, E. Zeitler, and A. Glick; the effect of beam potential on these processes was discussed by A. van Dorsten and A. Wilska. For biological materials (amorphous) the tendency has been to settle at approximately 50-kv beam potential although there is some promise of higher contrast at lower voltages (Wilska). There appears to be little reason to use beam potentials above 100 kv for general microscopy. However, in metallurgical applications, where crystalline specimens are the rule, there is considerable interest in experimenting at 500 kv and higher.

Quantitative Evaluation

A two-dimensional image, such as an electron micrograph, can often provide significant data without being subjected to quantitative analysis. There are many applications where the electron microscope can be used in qualitative fashion to good effect (for example, the observation of shape, condition, and texture of cells and tissues). In addition, however, the electron image contains a store of information which can be extracted by quantitative procedures. Methods of particle size analysis were discussed by W. Stoeber and the measurement of dimensions in the image plane was dealt with by P. Highton. Counts of particles, statistically analysed, can yield useful numerical data; the number of virus particles per unit volume of a suspension can be determined when specimens are prepared in a reproducible fashion such as by centrifugal sedimentation onto a substrate (G. Sharp). J. Reisner outlined the instrumental problems associated with the accurate determination and maintenance of magnification calibration which is not usually an easy matter because of the many instrumental variables involved. G. Bahr discussed the practical aspects of using calibration standards for various magnification ranges and reported on experimental errors of reproducing and determining absolute magnification values. Methods of measuring length in the third dimension by methods such as shadow casting or stereoscopy were outlined by J. Helmcke. Another kind of measurement one can obtain from electron micrographs is the absolute intensity from point to point in the image. This is a function of the interaction of the electron beam with the specimen structure and can be related to it. We can obtain absolute or relative values of mass per unit area of amorphous materials or of random microcrystals; the determination is relatively independent of atomic number or chemical structure of constituents. We can also determine the mass of a population of such particles in a whole field in one operation by the use of an ingenious optical system devised by Bahr and Zeitler. The transmission of electron beams through single crystals is more complicated and depends on their orientation. However, experimental studies of contrast related to mass thickness of these systems are encouraging (L. Reimer). Quantitative analysis of cut sections can be assisted by the incorporation of x-ray diffraction data (R. Burge) and by the use of mathematical or statistical techniques (F. Chayes and A. Loud).

Chemical Information

For the beam voltages and lens apertures usually employed in electron microscopy, image contrast for a given mass thickness (the product of density and thickness) is fairly independent of atomic number. This is advantageous for mass determinations but discouraging for chemical analysis. Cosslett considered the characteristic scattering of different elements such as carbon and gold. While the total effect per gram is about the same, there are significant differences in proportions of elastic and inelastic components and in their angular distribution. There are also differences in the voltage dependence of these phenomena. However, the possibility of utilizing these effects for chemical analysis does not appear encouraging. The most promising approach to chemical identification appears to be analysis for x-ray emission from regions irradiated with a small electron probe. It remains to be seen whether this scheme has enough sensitivity for specimens as thin as those normally viewed with electron microscopes.

The identification enhancement of chemical elements or groups by the attachment of heavy stains (positive staining) is an established procedure and its exploitation has not been fully realized (M. Beer). Microanalytical reactions can be adapted to electron microscopy for the identification of mi-

nute amounts of certain elements (E. Wiesenberger).

In negative staining one attempts to delineate structure by surrounding it or infiltrating its pores with nonreacting, high contrast substance (R. Horne and A. Glauert) but anomalous effects have been reported which may confuse the interpretation (K. Meyerhoff). In mixed systems with several components of different chemical and physical properties it is not always obvious what sort of chemical interactions or physical effects predominate and produce the intensity modulations observed in the image. L. Bachmann presented quantitative aspects of autoradiography in electron microscopy and dealt particularly with the properties of recently developed emulsions.

Electron Beams

Materials exposed to electron beams may suffer change or injury due to (i) radiation damage, (ii) heat, and (iii) the deposition of "contamination." The contamination is caused by breakdown products of residual vapors, mostly hydrocarbons and pump oils in the vacuum chamber. Three speakers discussed (L. Reimer, K. Kobayashi, and G. Bahr) irradiation changes in organic and inorganic materials. These consist of changes in form or crystallinity, alterations of chemical structure (for example, cross-linking of high polymers), ejection of atoms and groups of atoms from the target, and chemical interaction between residual vapors and the specimen. For a given resolving power in a recorded image a certain number of electrons must pass through each resolvable picture element. This has a bearing on the possibility of examining living cells with high resolution. H. Heide calculates that for a resolution of 150 Å and a 100-kv beam the dosage would be 10^6 roentgens. When compared with known lethal doses this makes it very unlikely that observations can be made at interesting resolving power with nonlethal dosage.

Reimer described ingenious experiments whereby he was able to determine quantitatively the temperatures of small areas of thin films as a function of beam parameters. This was accomplished by depositing small areas of metals such as indium (melting point 136°C) on the films and observing the radiation conditions producing their transition from solid to liquid state. The results are in good agreement with work by Cosslett who in a quite different approach measured the energy lost in specimens through analysis of the velocity spectrum of transmitted beams.

The contamination problem was discussed by H. Heide, R. Hartman and A. Wilska. Heide analyzed the residual vapors and found that the primary effect is due to hydrocarbons which break down and deposit on the specimen at a rate which in most microscopes is probably about 10 Å per second. This causes rapid deterioration of contrast. If hydrocarbons are removed, water vapor (mostly from the photographic plates) becomes the chief offender. The water molecules become ionized by the beam at the specimen and oxygen reacts with the specimen. Biological specimens which are largely carbon will be etched away. Residual vapors can be trapped and kept from the specimen but this is not generally feasible with existing microscopes.

Hartman described extensive experiments designed to measure contamination sources with a commercially produced electron microscope. He concluded that it should be possible to achieve an instrument virtually free from contamination by replacing conventional gaskets, using a titanium getter pump, and adding adequate cold traps. The main problem anticipated is that of getting a fresh batch of photographic plates down to operating pressure in an acceptable period of time.

Besides its effects on the specimen, contamination is also a major problem in that it deposits on the very small physical apertures (10 to 50 microns) used to direct the beam at the objective lens; this causes serious deterioration in image quality. It has been known for some time that deposition is prevented if the aperture is kept heated to about 240°C but it is difficult to bring in heater current without creating a distorting field. Wilska described the manufacture and operation of a hot aperture and baffles for keeping contaminating molecules from reaching it.

Preparation of Specimens

The rapid strides in the past 10 to 15 years in the application of electron microscopy to the study of cells and tissues have been truly remarkable. The cytological literature is growing beyond measure with reports on all kinds of materials. High resolution has been attained, mostly better than 100 Å and frequently as good as 10 to 20 Å. When however, as D. Parsons pointed out, one considers the problem of operating on these complex and fragile materials so that the micrographs obtained may be understood in quantitative fashion and related to the original material, the task appears formidable. "We require a fixative which will stabilize (by cross-linking or otherwise) a large variety of proteins, phospholipids, nucleoproteins, etc., to the dehydrating solvents and embedding materials. We also require it not to precipitate substances normally soluble in intracellular fluid. We expect it not to alter the conformation of individual macromolecules, e.g., by breaking -S-S- linkages or weakening hydrophobic bonds) since conformational changes will probably produce an alteration in structure at a grosser level." He concluded that it is probably too much to hope for a perfect fixative for whole cells. Ribosomes are seen with OsO_4, but not with permanganate. On the other hand there is evidence that permanganate fixes membranes better than OsO_4. Fragile components of disrupted cells can be handled and treated to advantage in surface films on a Langmuir trough, but they must still be treated to enhance the contrast of their structural components. Many features and effects of positive and negative staining techniques are still poorly understood. What we see after treatment with heavy metals is for the most part the metals themselves and these in most cases bear unknown relations to the original material present. Parsons raises the question: Is metal staining really necessary? Perhaps one should make efforts to obtain maximum contrast with unstained materials through instrumental improvements and in particular through the application of phase contrast technique in analogy with light microscopy.

S. Bullivant discussed freeze-drying which as a fixative step has the advantages of taking tissues from fresh to dry state without chemical or surface tension effects. The staining of such dried material presents certain problems and possibly some advantages. In "freeze-substitution" material is dehydrated, stained, infiltrated with embedding material, and polymerized all at low temperatures. There is some evidence that this may preserve fragile systems better than conventional methods.

W. Stoeckenius showed high resolution micrographs of sections of osmium

tetroxide-fixed lipids and discussed their interpretation. G. Schidlovsky described effects of stains and fixatives in various suspending media on the microscopic appearance of sections of two-layered systems: chloroplasts and artificial multilayers of fatty acids. With chloroplasts the results indicate that correlation between image density and specimen structure can be "normal," "intermediate," or "reversed," according to complex conditions present during fixation and dehydration steps. Lightness and darkness in the image can also be affected by the level of focus due to phase effects. The artificial multilayer experiments demonstrate fixative action of OsO_4 on saturated fatty acids without the production of noticeable contrast. They show bands which are double layers of fatty acid soap molecules and a 2- to 3-Å layer of barium atoms bound at the carboxyl groups within a double layer of fatty acid soap molecules. He concludes that band thicknesses of artificial multilayers, when measured statistically, relate well to chain length but that similar measurements on biological systems and the meaning of the lightness or darkness of the bands should be used with caution.

Experiments on the chemical reactions of OsO_4 and $KMnO_4$ (both widely used in preparing tissues) with amino acid peptides and proteins were reported by T. Hake. The results seem to prove that these reagents will render only a weak contrast in electron microscopy. They are often however used with $Pb(OH)_2$, $UO_2(CH_3COO)_2 \cdot 2H_2O$ and with glutar and adipindialdehydes. He believes OsO_4 is used with wide success because it preserves structure of tissues without the need of prefixation and because there are plenty of unsaturated compounds almost everywhere in the cell with which it can react. D. Peters showed the effects of a variety of fixatives on the appearances of virus particles in thin sections.

A brief session on an important aspect of electron microscopy, namely the characteristics of photographic materials and their use in recording electron images, was dealt with by E. Zeitler and R. Valentine.

A fundamental difference between the recording of light quanta and electrons is that in the latter case only one electron hit is required to render a photographic grain developable and one electron can hit several grains. This means that as we examine smaller and smaller areas of an exposed and developed emulsion we reach a point where the number of electrons causing the photographic density within the area is sufficiently small that statistical fluctuations or "noise" will be observed which is unrelated to specimen structure. Significance cannot be attached to areas of less than a certain diameter which is given by probability theory; the corresponding limiting dimension in the specimen is simply this diameter divided by the linear magnification. Valentine estimates that for visual observation on a fluorescent screen we need a current density at the screen of about 4×10^{-11} amp/cm^2. If we are to record the image at this intensity in a reasonable exposure time, say 1 to 4 seconds, the number of electrons per unit area of the plate is fixed and the probability theory gives a dimension below which one is observing noise (or grain), not specimen structure. He examined a wide variety of photographic materials in a variety of developers and produced a table which gives the minimum magnification required to show prescribed specimen detail for each emulsion. The magnification depends to some extent on the nature of the specimen being about 3 times greater for arbitrary biological materials than for heavy metallic particles. For 10-Å resolution and plates commonly used, the magnification should be about 50,000 or more.

This symposium was sponsored by the Armed Forces Institute of Pathology and the Intersociety Committee for Research Potential in Pathology. The proceedings will be published in book form as soon as possible.

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Organic Photochemistry

The dynamic character of photochemistry was demonstrated by the International Symposium on Organic Photochemistry held in Strasbourg, 20–24 July 1964. The symposium was preceded by a 3-day course of lectures on the elements of photochemistry which was attended by a smaller number of students. The latter group included many young investigators who are initiating research in the field.

The meeting showed very clearly that photochemistry, despite its enormous scope, is a field having genuine integrity. Participants known ordinarily as theoretical chemists, physical chemists, and organic chemists repeatedly found common ground for vigorous discussion. Exciting new information and understanding seemed to whet the appetites of the symposium members for more insight into remaining vast areas of confusion and ignorance.

The symposium program consisted of 15 principal lectures and two sessions devoted to presentation of brief communications. Since the latter programs were only organized after the meeting was convened, many of the brief reports dealt with work currently in progress in the laboratories of the investigators. George Hammond (California Institute of Technology) and Jacques Levisalles (Strasbourg) organized and directed the meeting.

Subjects of the lectures involved new photochemical reactions of both large and small molecules, direct study of excited states, various systematic schemes for visualization of the chemical reactions, and nonradiative decay of excited states. The impact of the concept of excitation transfer in photoreactions was illustrated by Paul de Mayo's (Western Ontario) classic remark, "So, wishing to know what was going on, we reached for a sensitizer." Even the inverse of photochemistry, chemiluminescence, was discussed (E. J. Bowen, Oxford).

The keynote of the conference was provided in the opening lecture by W. A. Noyes, Jr. (Texas) who discussed "The problem of energy dissipation by excited states," a matter to which he has personally given many years of study. The struggle of electronically excited molecules to divest themselves of excess energy is, of course, the real story of photochemistry. The chemical changes only represent special cases of radiationless decay processes. The subject of relaxation of excited molecules recurred repeatedly during the conference and was the principal subject of a paper by H. M. Frey (Southampton) who discussed the chemistry of "hot" products produced by reaction of methylenes (from photolysis of diaziranes) with various substrates.

Not surprisingly, the photochemistry of ketones and aldehydes was the main subject of no less than seven lectures and was mentioned frequently in other discussions. A considerable amount of order seemed to emerge. Apparently most carbonyl compounds undergo intersystem crossing very efficiently,