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Some Recent Developments in the Field of Electron Microscopy

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IT IS A COMMONPLACE TO SAY that experimental science is the systematic observation of nature and the attempt to set the observations thus made into an interrelated and logically consistent scheme. For this, contact with the outside world is through our senses, with vision bearing the overwhelming burden. When, in its early days, physics was concerned with the properties and behavior of matter in bulk, the unaided human eye was usually an adequate instrument. But the moment science began to occupy itself with the fine structure of matter, increase in knowledge became intimately geared to our ability to design and build instruments that would supplement, enhance, and refine our perceptions. It was thus inevitable that in penetrating more and more deeply into the structure of matter the path between what is finally perceived and the phenomenon of nature that initiated the perception should become more tortuous and the intellectual chain involved in its interpretation more complex. While it is undoubtedly a great intellectual satisfaction to devise and operate successfully such complex approaches to the secrets of nature, we sometimes lose sight of the fact that this complexity is not in itself desirable, that it inevitably limits the range and clarity of our view, and that information derived in a circuitous fashion is always come upon the hard way and is increasingly subject to error. Therefore, anything that cuts the path between what is seen and what originates the perception is bound to broaden and simplify our understanding. The electron microscope is of the greatest importance simply because it has made unexpectedly direct our perception of a world of things which we have felt certain existed but knew to be too small ever to be seen with any form of light.

It is hard to realize the great extent of the microworld thus opened up. Several investigators have worked for a generation in developing an ultraviolet microscopy that cannot reveal particles smaller than half those to be seen with visible light. In theory, the electron microscope can delineate particles a thousand times smaller; and, though they are scarcely 10 years old, such microscopes have already extended our vision a hundredfold. In taking this step we span that range of organized matter which extends from the animate to the lifeless and which must be understood as a basis of what we intuitively mean by "living"; at the same time we acquire the ability to "see" the larger of the molecules that are the basic units of chemistry. It rarely happens that any new experimental technique allows as direct an approach to the problems of a single science as the electron microscope thus gives to the fundamentals of both chemistry and biology.

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Problems of Electron Microscopy

The problems of a developing electron microscopy are threefold. The first is concerned with the design of electron lenses and their combination to produce the best attainable microscopes. The second involves a careful and thorough examination of the fine structure of biological and chemical substances with these instruments. The third set of problems deals with the application of knowledge gained in this fashion to such practical matters as the size of the submicroscopic particles in smokes, pigments, and mine dusts, or the relationships between structure and properties in materials as diverse as metallic alloys and natural and synthetic fibers. The discussion that follows is devoted mainly to the second group of problems.

Those interested in the electron microscope as an approach to the microstructure of matter need have a less intimate preoccupation with its development than was the case a few years ago. Early experimentation with a variety of designs—for emission microscopes and scattered-electron, including dark-field, microscopes and for microscopy at various volt-

ages, to choose a few examples—has served to indicate pretty clearly the basic requirements of the kind of instrument needed to meet present problems. Also, commercial microscopes are already available which are far better than we could build if we had the facilities and inclination to try to do so. This does not mean that the existing instruments are ideal or utilize to the full the theoretical potentialities of microscopy with electrons, for it is inconceivable that an instrument so new and complicated will not undergo large-scale and perhaps revolutionary changes in design and construction as we learn better how to use it, what its rewarding uses are, and how best to make its lenses. It will indeed be a misfortune if such changes do not come about. But for those who are microscopists rather than specialists in electron optics, the important thing is that there is ready at hand an instrument whose possibilities have as yet been little explored. Too often scientific instruments become commercially available only after their most important potentialities for research have been exploited.

Types of Electron-microscopic Objects

The fundamental properties of electrons place certain restrictions upon what can profitably be examined in the electron microscope. Preparations must be looked at *in vacuo,* and they must be unusually thin. With this in mind, it is convenient to group objects for electron microscopy into three general classes: (1) sections cut from tissues and other materials; (2) ultrathin replicas of the surfaces of thick objects; and (3) suspensions of small cellular elements and of particles of colloidal and macromolecular dimensions. Each of these has its own problems of technique, and electron microscopy as a whole is still in that early stage of development where both the direction and the rapidity of its growth are determined largely by the evolution of these techniques.

Electron Microscopy of Tissues

Many promising biological and medical applications of the electron microscope depend on the satisfactory development of ways of cutting and handling the thin sections referred to under (1) and of differential stains for the sections thus prepared. For their micrographs to be valuable, biological sections must be less than .5 micron thick; sections from heavier materials will have to be still thinner. In the hands of very experienced operators the best available microtomes will cut down to .5 micron, but this involves pushing both instrument and skill to such a degree that other and novel procedures are obviously called for. The first to be suggested, by Von Ardenne, involved cutting wedge-shaped, instead of the usual flat, sections. He showed that this could be done and published illustrative pictures. This method appears to have been little used in Germany, and a trial along similar lines by Richards, Anderson, and Hance has not been followed up. In this country most of the emphasis has been put on the development of a microtome to cut ultrathin sections. Such an instrument, as suggested by O'Brien and McKinley and made in usable form by Ladd and Braendle and by Fullam and Gessler, is characterized by having a thin, instead of the usual massive, cutting blade put at the rapidly moving periphery of a rotor. It permits a totally new approach to many problems of cellular structure and is of the greatest importance for the future of electron microscopy. An enormous amount of careful work will be required to recognize many of the structures in sectioned tissue and to provide adequate interpretations of what is seen. Nevertheless, there are important problems that can be approached with assurance of immediately suggestive results. Thus, in spite of the discouraging early micrographs of sectioned muscle, the beautiful recent photographs of isolated tendon and muscle fibers by Schmitt, Jakus, and Hall demonstrate the extraordinary internal regularity that exists and that undoubtedly can be made apparent in satisfactorily sectioned tissues. Sections through other fibrous and elastic materials, such as natural and synthetic rubbers, and plastics, will inevitably throw light on both their inner structure and what happens during their formation. It is hoped that the electron microscope will eventually make clear the kind of organization that must be present within the nuclei of cells. Attempts to observe chromosomal structure have not yet been rewarding, but progress in this direction should follow their photography in section. Another problem that can now be attacked with great profit concerns the morphology and manner of growth of viruses within the cells they infect. Most virus preparations thus far examined with the electron microscope have been purified, in the centrifuge or otherwise. A knowledge of their morphology under these circumstances is a necessary but not a sufficient step, since procedures that purify are by their nature selective and ones that separate virus from the matrix in which it is produced. The electron micrography of sections through virus-infected cells must lead to a rapid increase in our understanding of how their particles are produced.

Stereoscopic microscopy will be especially helpful in bringing out the spatial relationships between details seen in sectioned material. Since its introduction in 1940 this technique has been used with striking results upon such diverse objects as small crystals, bacteria, diatoms, the wings and tracheae of insects, and surface replicas of metallographic preparations

and of teeth. The ingenious reproductions of these stereoscopic micrographs, using polaroid, are especially useful for teaching and demonstration purposes.

A recent paper by Porter, Claude, and Fullam introduces another promising way of studying tissue cells. This involves their growth in culture under conditions giving single-cell spreads thin enough for direct electron photography. The pictures already published show that, where such extended sheets of cells can be grown, they yield excellent and instructive micrographs. This is another technique applicable to the problem of how viruses grow and multiply.

Electron Microscopy of Surfaces

Several ways have been devised for the electron-micrographic examination of surfaces. In early work the surface being studied was made a source of electrons which were condensed into an "image" of the surface. Depending on the material being investigated, the electrons could be either photo- or thermally emitted, or they could be electrons from a secondary source after differential scattering from the surface in question. Though these procedures have been, and will continue to be, helpful in the solution of such special problems as the electron emissivity of tungsten or oxide-coated surfaces, they have an obviously limited applicability. Closely related to the scattered-electron type of microscopy is the "dark-field" microscopy which forms images from the electrons scattered on transmission through a specimen. The German literature contains many "dark-field" pictures. Some are striking in appearance, but they do not seem to give as much information as corresponding bright-field photographs, and it is not certain how much use can be made of them.

The fine structure of the surfaces of many solids can be investigated by preparing replicas thin enough for transmission microscopy. The first replicas of this sort were described by Mahl and by Koch and Lehmann in 1940. In this country replica work was initiated by Zworykin and Ramberg. Early replicas were made of shellac, collodion, and other plastics, of silver and beryllium, and of oxide produced by anodizing an aluminum surface. The polystyrene-silica method of Heidenreich and Peck and the use of formvar by Schaefer and Harker advanced the art by providing convenient ways to study surfaces without damaging them. Formvar and other simple plastic replicas produce relatively little contrast in their micrographs and therefore reveal little of the fine detail they may contain. More contrast is obtained by the polystyrene-silica method, but these replicas too have certain limitations: (1) Since a polystyrene cast is formed at elevated temperatures and pressures, it has a restricted application to many biological materials or to other objects whose position, form, or composition interferes with making such pressure casts; and (2), though silica gives better contrast, its apparent migration after deposition often makes equivocal the interpretation of fine detail.

Collodion replicas have not been much used until lately because little sharply contrasting detail can be seen directly on them. It has generally been assumed that this is due to the fact that collodion does not give a faithful small-scale reproduction of surface detail. Metal shadowing, as developed over the last two years by Williams and Wyckoff, has indicated that this is not the case but that, on the contrary, properly made collodion or formvar replicas reproduce fine structure all the way down to molecular dimensions. Since plastic replicas formed from solution are especially easy to prepare and can be taken from fragile and relatively inaccessible surfaces, and since metal shadowing brings out the detail on them, such shadowed collodion or formvar replicas are applicable to many problems. Evidently techniques are now at hand for teaching much that is new about the surfaces of metals, etched for metallographic analysis, machined in various ways, corroded, or worn by use; of glass and ceramics during and after a variety of chemical and physical treatments; of crystals found in nature or prepared in the laboratory; of many biological structures, such as large cells, microorganisms, and teeth; as well as of macromolecules distributed over a relatively smooth surface.

Electron Microscopy of Particulate Suspensions

To many, the overwhelming attraction of the electron microscope lies in the unique opportunity it offers for actually seeing big molecules. Partly for this reason and partly because relatively simple techniques of observation are required, much effort has already gone into the study of suspensions of small and submicroscopic objects. Information has been sought about the size distribution in many inorganic colloidal materials, such as gold and other metallic sols, clays, carbons, pigments, dusts, and smokes, and many practically helpful results have come out of this work.

Suspensions of microorganisms, like colloids, have been the subjects for numerous electron micrographs. Bacteria were among the first objects to be examined, a first photograph having been published by Marton as early as 1937. The morphological survey of various bacterial species which has followed, and which is as yet far from complete, is a necessary preliminary to other uses of the electron microscope by bacteriologists. Some have expressed disappointment with

what the microscope has shown about bacteria, and there have indeed been no exciting discoveries about the fundamental properties of their nuclear and protoplasmic structures. Perhaps the inevitable improvements that will come about in ways of handling and preparing these relatively large objects will sometime give sensationally interesting results, but whether this happens or not, sound morphological studies, repeated if necessary with each advance in technique, are necessary. This is equally true for other microorganisms, both larger and smaller than bacteria. There have been investigations of spirochetes, such as those of syphilis and infectious jaundice; but serious studies of many other interesting large microorganisms, such as molds and the malarial parasite, remain to be made. Nor has much yet been published about the especially small organisms, like the highly pleomorphic causative agent of pleuropneumonia or the infectious units of trachoma, psittacosis, and related diseases, that lie between bacteria and viruses. Their very minuteness, which renders observation by classical methods so difficult, makes them particularly desirable objects for electron microscopy.

The electron microscope offers a new way to investigate the chemical and immunological reactions of bacteria and bacterial products. Except for photographs of the capsules of pneumococci and of bacterial cellulose, the products of bacterial metabolism remain to be investigated; and only preliminary papers have appeared dealing with the potentially fruitful field of the mechanism of the action of antibiotics and germicides on bacteria. Electron microscopy is a direct approach to the mechanism of the reaction that occurs between an antigen and its antibody. Here again, existing work is of a preliminary character, being restricted mainly to a demonstration that the reaction is discernible in electron-microscopic preparations. Thus, Mudd and Anderson have shown the change in appearance of typhoid and other bacteria in the presence of their antisubstances, the apparent thickening of flagella by flagellar antibodies, and the capsular swelling that takes place when a pneumococcus is mixed with its antibody. For the improvement of vaccine and serum production, as well as for more fundamental reasons, it is important to separate and purify from one another the various antigens associated with a bacterium. The ability of the electron microscope to make evident macromolecules means that the larger of these antigenic components can be photographed; Shepard and Wyckoff have in this way "seen" what is presumably the soluble antigen of typhus and its reaction with antirickettsial serum. This method of study can be applied to many problems in bacteriology.

A most interesting group of isolated objects larger than bacteria are not microorganisms but suspensions of cells and tissue elements, of both plants and animals. In connection with a study of the clotting of blood, early photographs of platelets and of the walls of erythrocytes were published. Claude and Fullam have photographed spherical elements from the cytoplasm of leukemic cells from the rat which they considered to be mitochondria. Preliminary pictures have also been taken, both in this country and abroad, of the analogous chloroplasts from plant leaf cells. Spermatozoa have been photographed and their tails found to have a fibrillar structure. An early examination was made of the fine details of bird feathers and fragments of the chitinous shells of insects. Especially suggestive for further work is the observation of a fine structure, resembling that of collagen, in photographs of iridescent insect scales. But this was not found in nerve axoplasm of the squid, nor has it been seen yet in isolated chromosomes and other nuclear material from cells.

The most impressive demonstration thus far of structure within tissue elements results from the study by Schmitt and his co-workers of separated phosphotungstic acid-stained fibers of tendon and the coordination of this electron-microscopic structure with the X-ray diffraction effects it produces. This regularity, independent of width, is seen in fibers whose cross-section grades downwards to the limit of visibility with present-day electron microscopes. Evidently we are dealing here with elements that approach true fibrous protein units. Electron-microscopically visible structure will doubtless be found in other fibrous proteins. There is evidence for structure in fibrin, and it is clear in the micrographs of separated muscle fibers and trichocysts of paramecia. Only a start has been made in the electron-microscopic analysis of wool and cellulose.

Limits of Electron-microscopic Vision

Two factors dominate the photography of especially small particles and minute details of structure: contrast and resolving power. As already stated, the limit of resolution of existing microscopes is approached only when dealing with particles of molecular dimensions. Contrast is rarely a problem when photographing smokes, dusts, and elastics like rubber that do not have to be supported on membranes, nor is it often a limiting factor in the photography of inorganic colloids. But macromolecules are organic and hence are composed of light atoms whose scattering power for electrons is low. There is so little matter in these molecules that, especially after being mounted on supporting membranes, they offer very slight contrast; in practice the lower limit of what can be seen clearly is set by such lack of contrast

rather than by insufficient resolving power in the microscope. For the satisfactory observation of macromolecules and of molecular detail in organic material it is therefore necessary to have recourse to a staining or impregnation that will enhance contrast. Two ways of doing this suggest themselves. One, to bring out internal structure, is analogous to the customary staining of biological material except that a "stain" for electrons is a substance of great scattering power. Such chemical staining was involved in the treatment of collagen with phosphotungstic acid; it occurs when this or some other protein coagulant—or a relatively heavy ion, for instance—is added to a virus preparation. Williams and Wyckoff have found that metal shadowing, involving the oblique deposition of heavy atoms that do not subsequently migrate over the preparation, also acts as a surface stain to increase the visibility of macromolecules and bring out their shapes. Müller, in Germany, like ourselves, tried the oblique evaporation of metal as a device for measuring the heights of electron-microscopic objects; but he worked with silver which does not give the continuous, essentially grainless, film needed to show fine detail and to cause the three-dimensional impression we have found useful in outlining particle size and shape. Mahl also tried evaporating chromium onto replicas, but there does not appear to have been any follow-up of this type of work in Germany.

At present it is impossible to say exactly what is the smallest particle that can be recorded clearly in electron micrographs. This question is obviously of vital concern to all who are occupied with the photography of molecules. Resolving power of an optical system is commonly defined in terms of its ability to show as separate two objects exceedingly close to one another. A picture published by Von Ardenne contains two particles of colloidal gold separated by not more than 40 A.; in another photograph, by Von Borries and Ruska, inorganic particles appear separate, though the distance between their centers cannot exceed 25 A. Von Ardenne has called attention to an image which he ascribes to a particle no more than 10 A. across—apparently the smallest anyone has yet claimed to have seen with the electron microscope. In studying molecules one is more interested in the ability of the microscope to provide a faithful image of a molecule than in mere indications of its presence. It is not simple to tie this up with such a definition of resolution as the foregoing, but more will be known about this after photographs have been made of several molecules of established shape. Even if the foregoing represents a somewhat optimistic estimate of the powers of the average microscope, we are now in a position to investigate most proteins, both globular and fibrous; many synthetic long-chain polymers; polysaccharides including dextrins, starches, and cellulose; and the world of viruses that reaches upward to the lower limit of visibility of the optical microscope. With the means now at our disposal it is altogether probable that many new molecular entities will be discovered within this region.

Photography of Macromolecules and of Viruses

Attempts to photograph macromolecules began early in the history of electron microscopy. The first molecule-like particles to be recorded were tobacco mosaic fibrils, which Kausche and Ruska showed to be the elongated fibers now familiar to all. This and other early work on plant viruses by the German school was followed by photographs of molecular suspensions of hemocyanin, edestin, glycogen, and an iodine-containing reaction product, *p*-iodobenzoyl glycogen. Pictures of hemocyanin and edestin molecules, by Stanley and Anderson, were the first to appear in this country. Since then, excluding studies of rubber, collagen, and polymers, whose fibers are not molecules in the sense used here, there has been only a limited extension of molecular photography.

Williams and Wyckoff have recently shown that metal shadowing permits considerably improved molecular photography; and the practical lower limit of size of molecules that can be distinguished is now set by the particulate structure of the collodion or other substrate. To work with the smaller molecules, then, this collodion structure must be minimized or a smoother substrate found. Formvar is not much smoother, but we have found that its structure or that of collodion can be largely suppressed through recourse to a replica technique wherein molecules on a smooth glass surface are shadowed and the resulting film backed up with unshadowed plastic. Such replicas have already delineated molecular particles at least as small as 100 A.

These experiments should be extended in two obvious directions. One is concerned with photography of smaller and smaller molecules; the other involves photography of the products of reactions between macromolecules or of the substitution of heavy atoms into such molecules. The reaction between an antigen and its antibody is the best known, and certainly one of the most important, intermacromolecular reactions; others deal with the adsorption of colloidal particles onto macromolecules, and the polysaccharide-protein, lipoid-protein, and nucleic acid-protein combinations that are so common in nature. It would be rash to try to foretell what the photography of macromolecular reaction products will show, but obviously we have a new way of examining the denaturation of proteins and their change in molecular shape and size with changes in pH and under the action of such

splitting agents as urea; and it is not unreasonable to look forward to the time when we will be able to see directly the action of enzymes.

Shadowed molecular photography permits study of the structure of collodion and other reaction and split products of cellulose. In a similar way information about muscle will come from the photography of its extracted protein myosin. Such investigations of chemical derivatives will undoubtedly take their place alongside studies of mechanically disintegrated and sectioned material as approaches to the structure of fibrous substances.

Just as viruses are intermediate in size between molecules and microorganisms, so the problems they present are partly molecular and partly biological. Not only can the electron microscope define the sizes and shapes of elementary virus particles, but it also offers a way of examining details of the chemical reactions into which they enter and of seeking how they originate within their host cells.

Electron micrographs made of purified viruses show particles whose dimensions are in general agreement with the results of indirect physicochemical measurements. Through the work of Krause, Stanley and Anderson, Sharp, and others we now have micrographs of several plant viruses, the eastern and western forms of encephalomyelitis, the rabbit papilloma, several strains of influenza, vaccinia, and ectromelia. Photographs have also been made of infectious material from hoof-and-mouth disease, silkworm jaundice, human and murine poliomyelitis, chicken pox, and herpes. Partly because of the unusual sperm-like shape of the particles of some strains, bacteriophages have proved excellent material for electron microscopy.

The particles in some of the purified virus preparations are uniform in size; with others, size varies about a mean. Thus, the spherical particles of the bushy stunt plant and the papilloma animal viruses appear alike, their diameters being those already determined through ultracentrifugation. The spherical particles of such a virus as influenza, on the other hand, are far from uniform as seen under the microscope. It is sometimes suggested that such variation is evidence for a microorganismal, as opposed to an essentially molecular, character, but this is not a valid argument since similar variations in measured size occur among the molecules of a protein like hemocyanin. This nonuniformity in diameters presents an important problem. It may indicate that the molecules of a protein need not be all alike, or it may be a consequence of an irregular shrinkage or desiccation of particles strongly hydrated in solution. This will be answered when we have enough accurately measured micrographs of several kinds of molecules and a correlation of these measurements with particle sizes and degrees of hydration as determined by physicochemical analysis.

Important chemical reactions of viruses are those in which they are inactivated, as for vaccine production, or are neutralized by specific antiserum. There has been no thorough microscopic study of inactivation, though treatment with formaldehyde is known to have little, if any, effect on the appearance of the elementary particles of some viruses. A splitting of the tobacco mosaic fibers by physical means has been photographed. Micrographs already made show that the reaction between virus and antibody is readily recognized: the virus particles appear bigger and more diffuse in outline, presumably due to attached antibody, and agglutination on a microscale is common. If certain current hypotheses are correct in suggesting that this combination involves the formation of antigen-antibody lattices, such regularity should be clearly evident. On the practical side the minute amounts of virus, or antibody, that go to make up a microfloc indicate that highly sensitive diagnostic tests can be built around the electron-microscopic observation of this immune reaction. Another reaction, chemical or biological, upon which microscopic studies have already been begun, is the lysis of bacteria by their bacteriophages.

The electron micrography of crystallizable viruses and proteins offers a direct approach to questions of how crystals are built up from their molecular units. Crystal formation can be photographed, and this will provide very precise knowledge of many of the factors that determine crystallization, of how microcrystals form and grow, and of how true crystallinity is related to the paracrystallinity observed with fibrous materials like the tobacco mosaic virus.

The problem of how the elementary particles of a virus come into being can now be investigated. Once these particles have been identified through work with purified suspensions, the road is open for dealing with less pure preparations, with the ultimate object of seeing viruses in the cellular environment in which they develop. Only a first step has been taken in this direction, but it has been recognized that filaments as well as spheres are present in purified influenza virus suspensions and that an intimate relation seems to exist between the two forms. Thus, we now have a way to see how viruses grow; and it is possible that these experimental procedures may ultimately tell how large "inanimate" molecules, like those of hemocyanin, originate. It is worth reiterating that with the electron microscope as guide and tool we are entering a world where, very literally, life begins. What will be found there, beyond useful information about viruses and the diseases they cause, can scarcely be foretold.