MEDICAL PRACTICE

Scientific Basis of Clinical Practice

Some Recent Contributions from the Electron Microscope Laboratories

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In the past few years many interesting developments have come from studies using the electron microscope. Some of these new methods and discoveries already have a clinical application, others offer a hope of clinical applications and developments in the future. This account will be restricted to work published since 1965.

Capabilities and Limitations

Though its appearance is impressive and its cost considerable, an electron microscope is basically simple: like an optical microscope turned upside-down, using electrons instead of light. The source of electrons is a V-shaped tungsten filament, which is heated to incandescence. Any incandescent filament gives off both light and electrons. In the conventional microscope lamp the light is used and the electrons just sit in a close cloud around the filament; in the electron microscope the light goes to waste and the electrons are propelled downwards by the application of a high voltage, usually between 25 and 100 kV. Electrons are soon stopped by air, so the microscope has to be evacuated to a vacuum of 10^{-4} torr. Instead of glass lenses, the electron beam is focused and the image formed by strong magnetic fields. Most electron microscopes have five or six lenses: one corresponding to the lamp lens, in the optical microscope, and one to the substage condenser; an objective, and a projector (corresponding to the eye-piece in the optical microscope); and one or two others, between the objective and projector, are used to vary the magnification—between about 500 and 200,000 times—of the final image on the fluorescent screen (Figs. 1 and 2). The high voltage and lens currents have to be very closely stabilized, to better than one part in 100,000: and it is largely this requirement for very high electronic stability, together with the requirement for the high vacuum, which make the instrument so expensive. Nevertheless, despite their cost, electron microscopes are now commonplace and no longer the status symbol they once were.

A limitation, overcome by the development about twenty years ago of techniques for cutting ultra-thin sections, is the poor penetrating power of electrons; to show fine detail the object must be less than 0·1 μm thick. Recently, megavolt electron microscopes of vast cost, with a beam capable of penetrating a 1 μm section, have become available, but, though useful to metallurgists, their contribution to biological knowledge has so far been small.

RESOLVING POWER

The resolving power of a microscope is limited to about half the wavelength of the radiation used. The wavelength of green light is about half the diameter of a staphylococcus and so limits the resolving power of the optical microscope to about 0·25 μm; about half this (0·12 μm) can be achieved with ultraviolet light and quartz microscope lenses. The wavelength of an electron beam is about 1/20th the diameter of a small atom, and so for practical purposes is not the factor limiting resolution in the electron microscope. Here the limiting factors are mechanical and lens current instability, spherical aberration in the lenses, and "noise."

All top-grade electron microscopes are now capable of an average resolving power of about 6-7 Å (0·7 nm) over the area of the picture (manufacturers' figures are based on selected fields and selected specimens on which much better resolutions can often be demonstrated).

Indeed, the resolution of single rhodium atoms has recently been claimed by Prestridge and Yates.[1] But even 0·7 nm resolution

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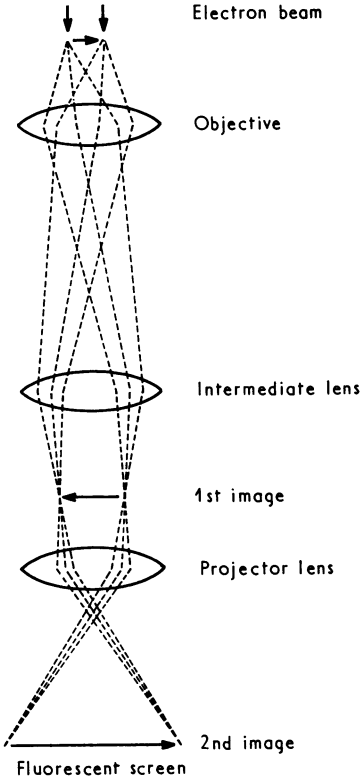
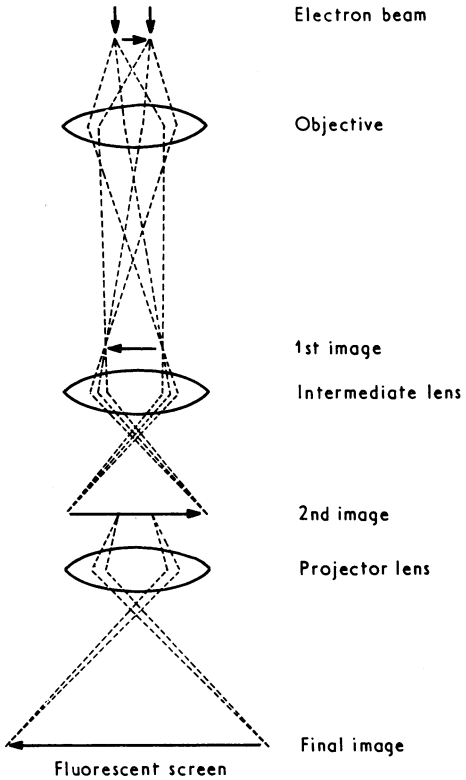
cannot be achieved with biological material, because the intrinsic contrast is too low; our bodies—and those of bacteria and viruses—are composed mostly of the lighter elements C, N, O, H, and a little P, some S, and some Fe. The Ca-containing compounds usually have to be dissolved out before thin sections are cut. 0·6 nm is only the length of a line of four carbon atoms and if we could command resolution of this order the coiling of polypeptide chains in protein molecules would be clearly visible. Very low voltage (4-6 kV) electron microscopes might allow higher contrast, and so better resolution of biological materials, but there are technical difficulties in making them and they have not been produced commercially. Even using negative contrast staining, about 1·2-1·5 nm is the best resolution we can get on biological material; and a useful resolution of better than 20-30 nm is rarely obtainable with sections.





FIGS. 1 and 2—Diagrams to illustrate image formation in the electron microscope at low and high magnification. Magnetic lenses have been symbolized as biconvex surfaces; they are not, of course, of this shape. The vacuum connexions and the electron source are not illustrated.

Diagnosis of Virus Diseases

When it was first invented it was hoped that the electron microscope would be as useful for the diagnosis of virus disease as is the optical microscope for diagnosing bacterial disease. But just as the application of optical microscopy had to wait for the invention of the right methods by Gram, Ziehl and Neelsen, Ehrlich, and others, so with the electron microscope useful applications had to await the right techniques. There is no Gram stain for viruses, or indeed for electron microscopy, but the invention of negative staining showed that some groups of viruses have characteristic and easily recognizable surface structures. Among these, fortunately, are the pox viruses and also the herpes viruses, including chickenpox and herpes febrilis. Electron microscopy had

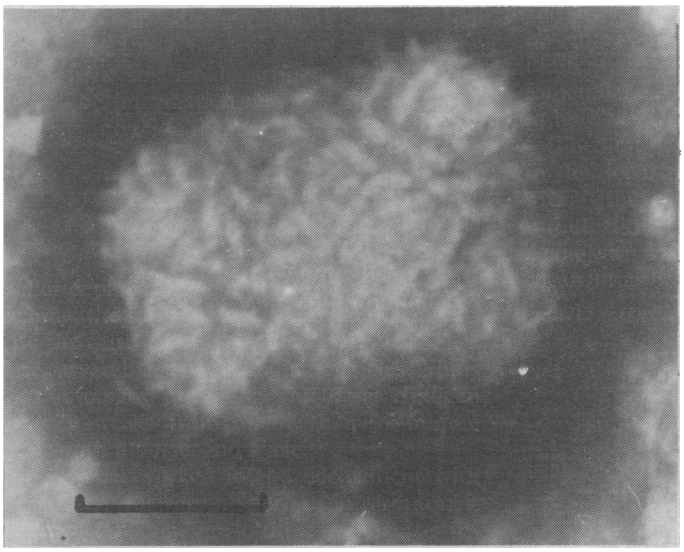


FIG. 3—A pox virus particle from a case of eczema vaccinatum. (Phosphotungstate × 240,000, bar = 0·1 μm)

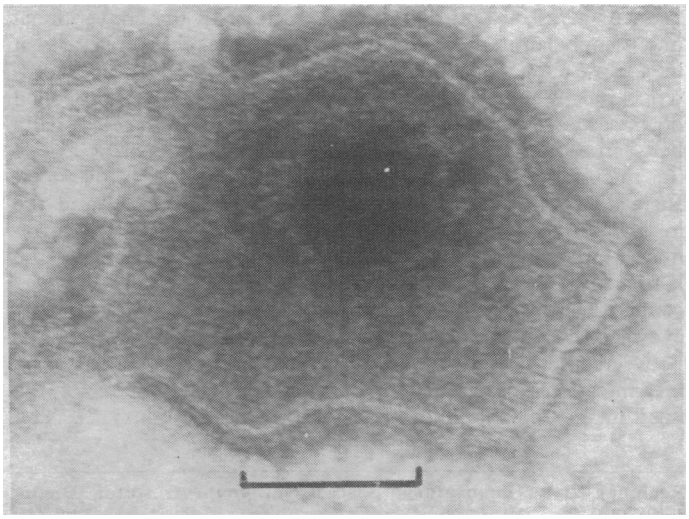


FIG. 4—Herpesvirus particles, from an early shingles lesion. (Phosphotungstate × 227,000, bar = 0·1 μm)

previously been used in 1962 for the diagnosis of smallpox, notably by Peters and his group in Hamburg[2] and a method had been proposed by Nagington and Macrae.[3] In 1966, however, when an epidemic of variola minor appeared in the Birmingham region a simple and rapid technique was used very effectively, not only to make diagnoses of smallpox, but even more importantly to exclude smallpox by making diagnoses of chickenpox.[4] The method is so simple that electron microscope grids could be prepared in a few minutes by the bedside in the patient's home. This was indeed done for the first few cases, but was soon found unnecessary; material spread on a slide and allowed to dry could be prepared for electron microscopy just as well in the laboratory.

Even from a financial point of view such speed in diagnosis is rewarding. During the 1966 outbreak a person recently arrived from abroad, with no vaccination scar, developed a rash of clinically doubtful nature in Stratford-upon-Avon, during the height of the tourist season. A confident diagnosis of chicken pox, with the aid of the electron microscope, saved three days waiting for the results of culture on the chorio-allantoic membrane, much anxiety in many medical minds, and a good many cancelled hotel bookings—no doubt the cost of several electron microscopes.

The method is applicable to crusts from both smallpox and varicella, or to dried smears kept in store for years after an epidemic (Figs. 3 and 4).

Other Virus Diseases

The virus of molluscum contagiosum is also easily detected; particles are found in enormous numbers in the cheesy material which can be expressed from the centre of the lesions. They resemble vaccinia and smallpox particles, but may be distinguished by the greater length of the protein filaments upon their surface. In practice, the clinical distinction between molluscum and smallpox is rarely difficult.

Subacute granulomatous lesions on fingers of people handling sheep may be due to the virus of orf, or contagious pustular dermatitis. In negatively stained material scraped from these lesions electron microscopy shows beautiful paravaccinia particles, each covered with a thread wound helically round it. They have a cross-gartered appearance in the electron microscope (Fig 5), because the electron beam passes right through the object, and as the depth of focus is considerable images of both sides of the object are superimposed. If the object can be tilted first one way, then the other, the successive pictures taken when viewed together give a stereo image and the two sides of a fairly thick object (like the orf virus) can be separated visually.

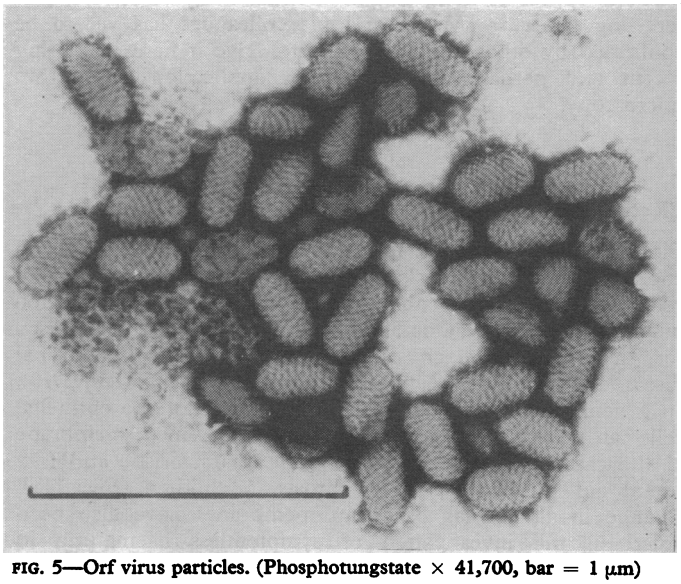


FIG. 5—Orf virus particles. (Phosphotungstate × 41,700, bar = 1 μm)

More recently, the electron microscope has been found of great value in the diagnosis and investigation of patients with serum hepatitis. Alone, of course, an electron microscope is only half a tool; other complementary techniques are required as well. An excellent picture of Australia antigen particles, found in the blood of patients with leukaemia, was published in 1966.[5] But the authors could not at the time interpret their interesting discovery, and the significance of the particles was not realized until some time later. But it is now well established that the presence of the particles of the Australia antigen is associated with serum hepatitis, and electron microscopy is as sensitive a method as most for detecting them. It has the disadvantage that it is much more time-consuming than serological methods; but no serological technique is immune from an occasional false-positive reaction, and the electron microscope is most useful for confirming doubtful positives. The appearance of the particles is characteristic enough (Fig. 6), but though one very small particle does rather resemble another their identity can be proved by showing that the particles are agglutinated by a standard reference

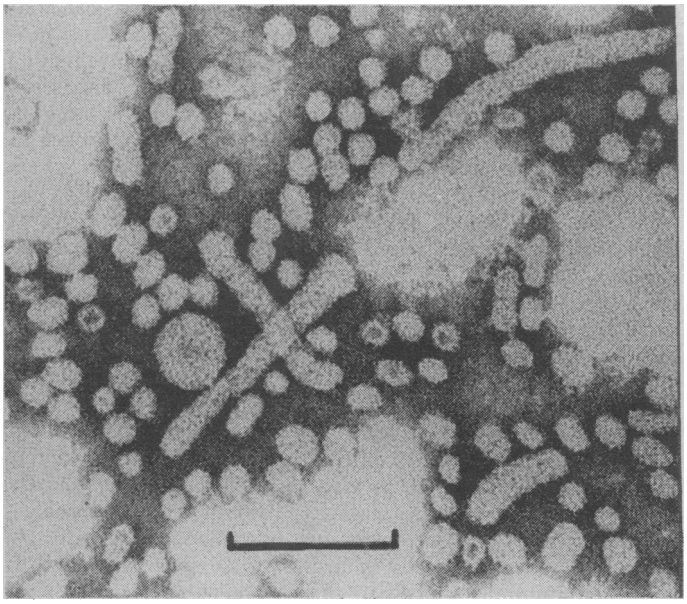


FIG. 6—Hepatitis antigen; small forms, large forms, and filaments are all present. (Phosphotungstate × 212,000, bar = 0·1 μm)

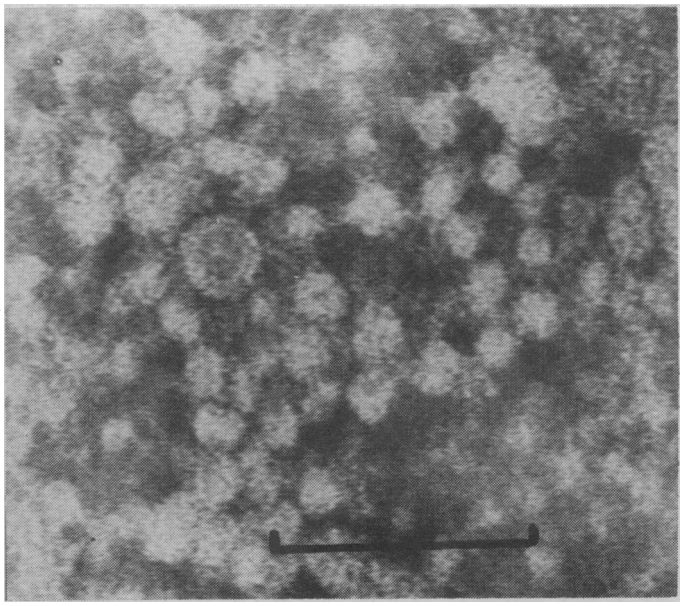


FIG. 7—Hepatitis antigen particles agglutinated by antiserum. The particles appear fuzzy from the globulin molecules attached to them and linking them together. (Phosphotungstate × 393,000, bar = 0·1 μm)

antiserum. This method was used by Blainey *et al.*[6] to confirm that urine concentrates, serologically positive for Australia antigen, did in fact contain particles which could be specifically agglutinated by antiserum to Australia antigen (Fig. 7).

This method of immune-electron microscopy was also used by Best *et al.*[7] to take perhaps the first definite pictures of rubella virus. When negatively stained this virus appears as a featureless blob, and only by attaching antibody molecules to them could the right sized blobs be identified. A refinement of the technique has recently been used to mark particular areas on the surface of foot-and-mouth virus particles by an antiserum specially prepared against a single protein component.

New Viruses

The existence of some new viruses has recently been discovered by electron microscopy and has led to two recent isolations. These new viruses may be related to human cancers, though it must be emphasized that no aetiological connexion has been established. In the rare condition of progressive multifocal leucoencephalopathy, a demyelinating disease seen as a late complication of malignant diseases—especially Hodgkin's disease and malignant lymphoma—abnormal large astrocytes are found in the white matter of the brain. These cells contain large vesicular nuclei, in which structures resembling virus inclusion bodies can often be seen. Particles closely resembling the carcinogenic polyoma viruses of rodents and the SV40 virus of monkeys were discovered in these nuclei by Zu Rhein and her colleagues in 1966; the same group have recently reported the isolation of a polyoma-like virus in tissue culture of human embryo glial cells.[8]

In the same journal, Gardner *et al.*[9] reported the isolation, using tissue cultures of "Vero" cells (a line of cells from African Green monkey kidney) of a very similar virus which they had detected in the urine of a patient bearing a kidney graft. (Figs. 8 and 9). This virus is biologically very closely similar to polyoma virus, though it differs from it serologically. It remains to be seen just how important this new virus may be. It is already well established that patients with grafts, on immunosuppressive drugs, are at a greater risk of lymphosarcoma than the population at large. Patients on immunosuppressive drugs are, of course, specially susceptible to all sorts of opportunistic viruses, and some of these might be carcinogenic, though none has yet been proved to be so.

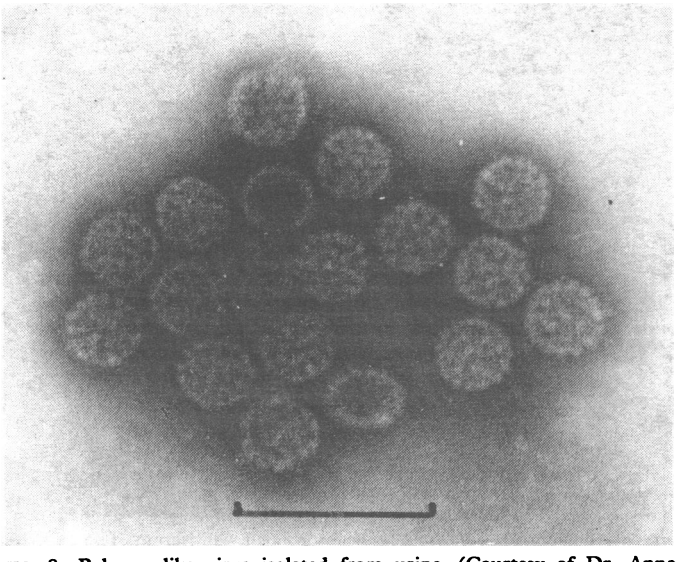


FIG. 8—Polyoma-like virus isolated from urine. (Courtesy of Dr. Anne Field.) (Negatively stained × 252,000, bar = 0·1 μm)

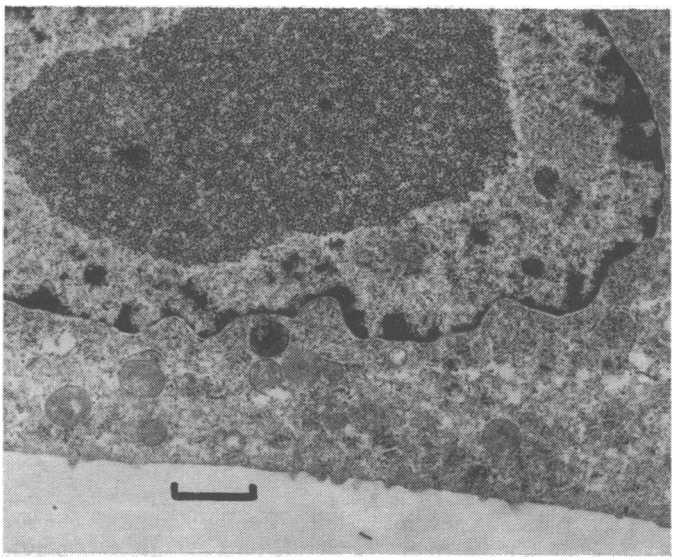


FIG. 9—Section through nuclei of ureteric lesion, showing intranuclear inclusion. Stained with uranium acetate and lead citrate. The inclusion is composed of great numbers of virus particles. (Courtesy of Dr. Anne Field.) (× 10,500; bar = 1 μm)

Cancer of the Breast

Small particles of all kinds abound in human cancer cells and many particles have been claimed as virus-like objects and possible causes of the cancer. But corroborative evidence implicating any particular particle has been lacking. Many other mammals do have their cancer viruses, notably mice. Among tumours caused by viruses, mice have a mammary cancer initiated by the virus discovered by Bittner, which is transmitted in the mother mouse's milk. Sometimes the milk contains enormous numbers of these particles. Female mice, if suckled on carrier mothers, get the cancer when they become sexually mature; so do males, but only when castrated and given oestrogens. "High-cancer" strains of mice exist, in which nearly all the females secrete the virus in their milk.

D. H. Moore *et al.*[10] have recently described virus-like particles, similar in appearance to the Bittner virus, occurring in milk and mammary tumours of Parsee women in Bombay. Parsees are an in-bred, self-contained community and have a significantly higher incidence of breast cancer than does the surrounding Indian population. These particles from human sources resemble closely those found in mouse mammary tumours, and sera from women whose milk contains these particles appear to neutralize mouse mammary virus. This interesting paper by Moore and his colleagues has yet to be confirmed by others, as I write, but I give it here as another recent and perhaps very important application of electron microscopy.

Electron Miscroscopic Histology as an Aid to Diagnosis

Electron microscopy of ultra-thin sections has yielded an enormous amount of basic information about the structure of cells and tissues, and about pathological processes—far too much to relate here. One application has, however, been used for the accurate diagnosis and hence treatment of renal disease: the electron microscopy of the glomerulus (Fig. 10). In a nephrotic glomerulus the foot-processes of the epithelial cells can be seen to have fused along the basement membrane of the capillary. This change is quite characteristic and is a useful aid to diagnosis of nephrosis in children (Fig. 11).[11] Change in glomerular basement membranes have also been noted in the investigation of symptomless haematuria in children.[12]

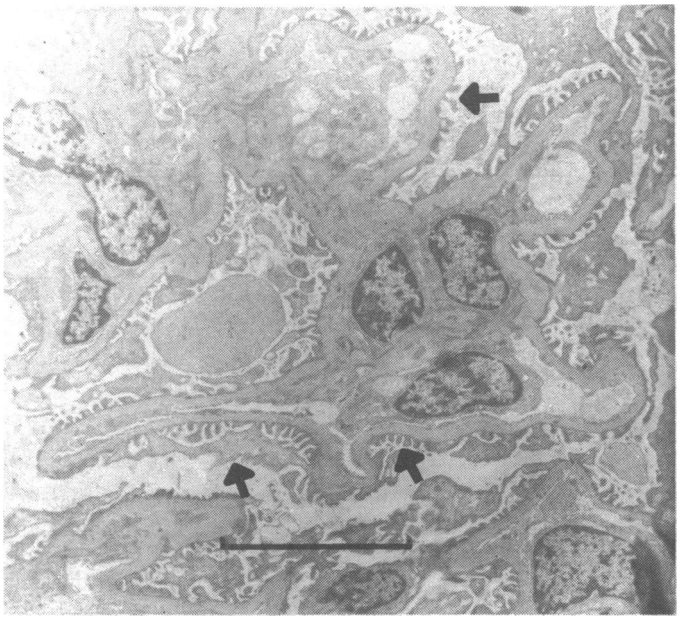


FIG. 10—Glomerulus, Henoch-Schoenlein nephropathy: no fusion of foot processes (arrows). (Courtesy of Dr. Eric Glasgow.) (Uranium acetate and lead citrate × 2,400; bar = 10 nm)

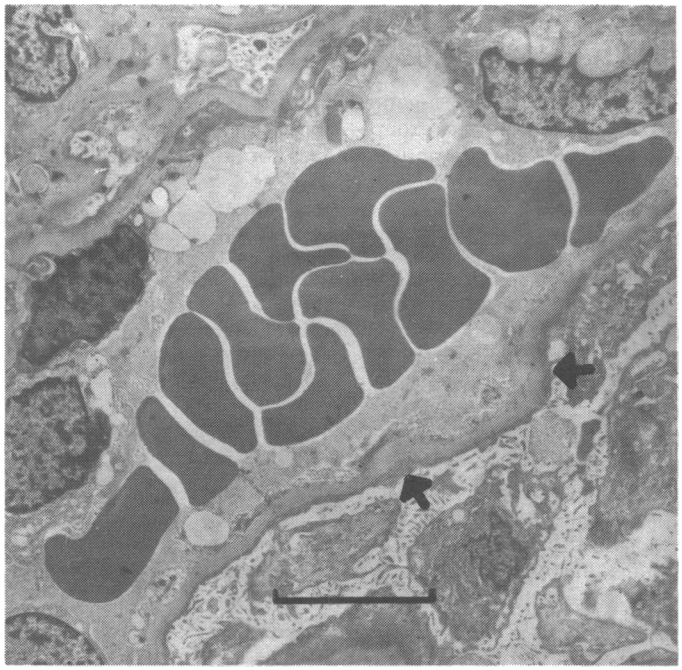


FIG. 11—Glomerulus, patient with nephrosis; fusion of foot processes (arrows) around a dilated capillary loop containing erythrocytes. (Courtesy of Dr. Eric Glasgow.) (Stained as Fig. 10 × 2,100; bar = 10 μ)

Scanning Electron Microscope

Solid objects can be studied, using the conventional electron microscope, by making replicas of their surfaces. To do this, a thin layer of plastic or carbon is evaporated on to the object, and the object itself is dissolved; or the applied layer—the replica—is stripped off and examined in the microscope. The technique is tedious, often difficult, and is time-consuming. It has the advantage that high resolution (1-2 nm) is obtainable on some specimens. But for specimens with very rough surfaces it is almost impossible. The scanning electron microscope is the answer. It works quite differently from the transmission microscope. A very fine beam of electrons from a heated tungsten filament is focused to a minute point and made to scan a small area of the surface of the specimen by magnetic scanning coils in just the same way as an electron beam is made to scan the face of a television tube. Secondary electrons scattered from the surface scanned by this pin-point beam are collected and amplified. This amplified current is used to modulate the brightness of a television tube, which is scanned in synchrony with the beam scanning the object in the microscope (Fig. 12).

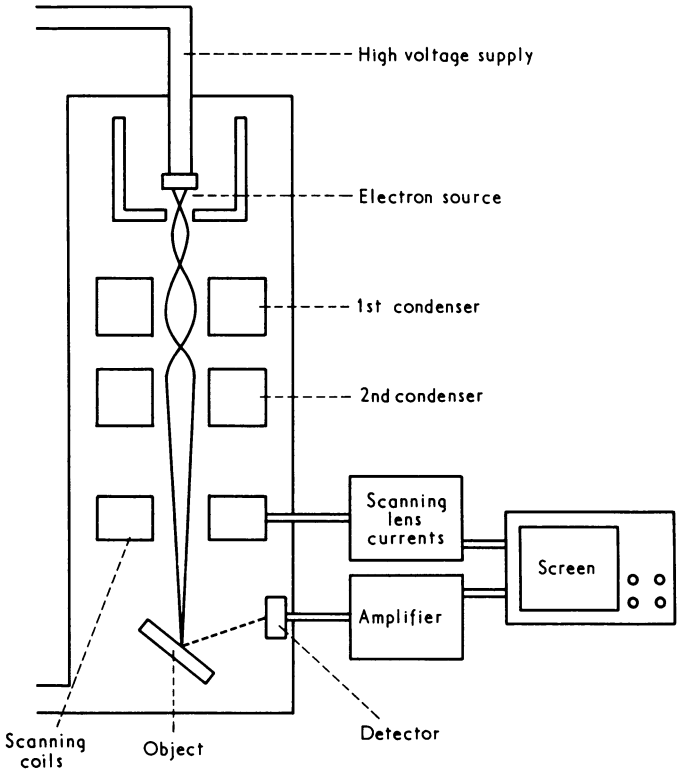


FIG. 12—Diagram to show general arrangement of the scanning electron microscope.

The result is a magnified image of the surface scanned; if the object it tilted one way and then the other, successive pictures can be viewed together to give a stereo image. And, in any case, the scanning electron microscope gives a strong three-dimensional effect, even in a single picture. Sometimes the pictures are most dramatic. Some recent applications have been in the investigation and diagnosis of blood diseases —abnormal erythrocytes are easily recognized by the scanning microscope.[13] Scanning electron microscope pictures have been published lately in many journals, so one is not reproduced here. Excellent examples appeared on the cover of the *Journal of Clinical Pathology* in November 1970, and in the same journal in November 1971.

This article is based on a lecture given in the Birmingham course under the title "The Scientific Basis of Clinical Practice" (see B.M.J., 27 November 1971, p. 510).

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