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Practical Aspects of Diagnostic Electron Microscopy

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The electron microscope technique of negative staining was first used to obtain fundamental information about virus morphology, but in recent years it has developed into a practical method for virus diagnosis. The methods employed are both simple and rapid. The following review discusses in detail the steps that must be taken to obtain good electron microscope preparations and illustrates some of the results that can be obtained.

The last decade has witnessed profound changes in a field that might be described as practical virology. In this context the word "practical" has two connotations: first, it refers to the techniques that are being employed for virus diagnosis, and second, it refers to the greater degree of action that can be applied in controlling individual episodes of viral infection and threatened dissemination. As an example, the newer techniques for detecting the presence of hepatitis B surface antigen (HBsAg), such as radioimmunoassay and passive haemagglutination, are technically simple and straightforward but offer a degree of sensitivity far in excess of such older techniques as immunodiffusion and complement fixation. The same example also illustrates the other aspect of the term "practical," for the recognition of a hepatitis B surface antigen carrier triggers one or more precise actions: the individual is banned as a blood donor, and, if hospitalised, he will be handled in such a way as to minimise contact with his blood. Yet more "practicality" will follow from the further development of viral chemotherapy [1].

During the last decade, as virology has moved from theoretical interest toward clinical application, the electron microscope (EM) has been an important tool for the basic understanding of viruses and, perhaps more important, for the rapid and unequivocal recognition of their presence. The achievements of the electron microscope in this era have been many and varied, and range from describing a new virus for the first time [2] to recognising the presence of smallpox virus in unexpected circumstances [3]. Unfortunately, however, an impression has developed that diagnostic electron microscopy is a capricious technique that works well only in the hands of experts. While this is not true, there are a few essential points of technique that do not seem to be widely appreciated; hence, the large numbers of technically poor micrographs that appear in the literature. This contribution will be concerned with a discussion of the practical steps that must be taken in order to produce a specimen that will yield good results in the electron microscope and a consideration of the means by which these results may be interpreted.

Only one method of preparing specimens for electron microscopy is suitable for

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rapid diagnosis and that is the technique of negative staining. Theoretically, this method is simplicity itself. The virus particle is surrounded by heavy metal atoms which act as an electron stain, the electron beam can pass through the low electron density of the virus but not through the metallic background, hence "negative stain," a light virus against a dark background.

Practically, the method is equally simple. A suspension of virus is mixed with a solution of the right kind of heavy metal ions, frequently those of phosphotungstic acid, and allowed to dry down onto a grid. As might be expected, a number of variations on this basic method have arisen and these will be described for the specific specimens to which they apply.

BASIC REQUIREMENTS FOR NEGATIVE STAINING

Negative Stains

There is a small number of negative stains, all based either on tungsten or uranium [4]. Probably the nearest to a universal stain is still phosphotungstic acid (PTA) which was the first negative stain to be described [5]. Since there are occasions when it is necessary to alter the proportion of stain to virus suspension, it is best to keep the phosphotungstic acid solution at a relatively high concentration. A 4 percent solution can be adapted for all uses and is the recommended strength. The pH of the stain is also of importance; for general use, pH 6 seems to give better contrast than either neutral or alkaline pH. However, some viruses, particularly foot-and-mouth disease of cattle and rhinoviruses, are acid labile and an additional phosphotungstic solution adjusted to pH 8 should also be available. In both instances the 4 percent phosphotungstic acid is adjusted to the desired pH by means of 1N potassium hydroxide. It is convenient to have the working solution of PTA in a drop bottle and to store the stock solution at 4°C.

Grids

Since negatively stained specimens are, by their nature, irregular in density, smaller size grid squares give greater stability. It is therefore recommended that 400 mesh copper grids be used. Again, because of the irregular nature of the specimens, the grid coating should be as tough and flexible as possible. Formvar-carbon has probably the best characteristics of available grid coating materials.

Disinfectants

Although not directly related to the method of negative staining, it is important to remember that negatively stained preparations retain biological activity. For example, when fluid is removed from the grid with a piece of filter paper, the paper is then contaminated and must be disposed of in a suitable manner. A container of hypochlorite solution, preferably with an anionic detergent, should always be present on the bench where negative staining is carried out.

Additional Items

Fine forceps, microscope slides, pasteur pipettes, and filter paper complete the equipment that is needed to carry out negative staining (Plate 1).

Types of Specimens

A distinction should first be made between specimens that *can* be handled and those that *should* be handled. Since the time investment for a single specimen is small, *any* unique or unusual specimen *should* be examined by negative staining. Since the amount of material required for the technique is also small, it is rarely, if ever, necessary to use all of a specimen for microscopy. On the other hand, large numbers of specimens of a routine or semi-routine nature *can* be examined but are time-consuming and frequently unnecessary. In the past only EM examination was available for identification of certain types of specimens, but newer techniques have been developed which are better suited for dealing with large numbers of specimens. A good example of this situation is seen in the hepatitis field. At one time, diagnosis of the hepatitis B antigen carrier state was always confirmed, if not indeed established, by electron microscopy. Now, radioimmunoassay and passive haemagglutination are the techniques of choice in reaching a diagnosis.

Almost any biological material will yield a specimen that can be examined by EM. The limitations of the technique are set by two main factors: the absolute amount of virus present in a specimen and the ratio of this virus to contaminating, or background, material. A concentration of 10^5 virus particles in urine will yield a good specimen as there is little contaminating material present; however, the same concentration of virus in sputum would almost certainly not be suitable because of background material. The absolute amount of virus required for success also varies with the virus type. For example, 10^5 particles of a small cubic virus would not be visualised, unless the more sensitive technique of immune electron microscopy (IEM) is used. This entails adding specific antiserum to the virus-containing specimen so that antigen-antibody aggregates are formed [6]. The small cubic virus present in the form of immune complexes is much more readily visualised than its unaggregated counterpart. Indeed, in an experiment carried out on low-grade hepatitis B antigen positive carriers, immune electron microscopy had approximately the same sensitivity as radioimmunoassay. Nevertheless, for the majority of viruses, a concentration of 10^6 virions/ml of starting material is needed if the particles are to be visualised by negative staining. Fortunately, both viable and non-viable particles are visualised in the EM and a specimen containing 10^6/ml physical particles may contain as few as 10^3 infectious virions per ml.

SPECIMEN PREPARATION

General Considerations

The final step in preparing a negatively stained grid is to allow specimen and stain, either together or consecutively, to dry down onto the grid. However thin, the layer has a third dimension and concentration of material occurs in the drying down phase. It is therefore of the greatest importance to minimise or eliminate any low molecular weight proteins and organic salts present. Organic salts appear as crystalline structures which ruin a specimen but are easily recognised; low molecular weight material dries down to an amorphous layer that can obscure a positive sample without the recognition that this has happened. Because of these considerations there are three basic rules that apply to the preparation of all specimens for negative staining.

1. The diluent employed is always distilled water. This has the advantage of lysing cellular structures but leaves viruses unharmed. It appears that virus particles are not sensitive to osmotic pressure.

2. Concentration by centrifugation should always be at the lowest possible speed. Virus is often entangled with fragments of cell debris and will be deposited at surprisingly low speeds. The large pieces of cellular material offer no problem in the EM and low molecular weight material will not be deposited. The duration and speed of centrifugation will vary somewhat with different types of specimen but many can be handled by centrifuging for one hour at 15,000 *g*. This speed might seem low, but the loss in virus recovery is more than offset by the improved quality of the final grid.

3. When the final pellet has been obtained, it is of the utmost importance that the last drops of fluid in the tube be removed. If fluid is present when the pellet is resuspended, low molecular weight material contaminates the specimen. The following procedure ensures that the tube is well drained. Decant the supernatant and maintain the tube in an inverted position; still keeping it inverted, place it in a beaker containing an absorbent tissue. Several tubes may be placed in the same beaker and, if there is to be some delay before microscopy can be carried out, the beaker can be sealed with either foil or parafilm and placed at 4° C. From a safety point of view it must be remembered that the tissue and beaker will become contaminated, and for this reason a small amount of hypochlorite should be used to moisten the tissue. When the tubes are removed from the beaker, still in an inverted position, the rim should be examined for drops of fluid which can be removed with a strip of filter paper. Only after this should the tube be turned the right way up and the pellet resuspended (Plate 1).

Methods of Negative Staining

1. *Standard Method.* The advantage of negative staining over thin sectioning is that the final specimen can be derived from an initially large sample, thus minimising sampling effects. Obviously, however, this large sample must be concentrated and, while several techniques are currently available, centrifugation remains one of the most efficient. The final pellet is resuspended in a small amount of distilled water, the exact amount of which is governed by the size of the pellet, which can vary from complete invisibility to several centimetres in diameter. The invisible pellet is suspended in a minimal amount of water, approximately 50 μl, while all other pellets are resuspended and diluted until they show only a slight opalescence. The main fault of beginners is to make the suspension too concentrated, and one suggestion that may be meaningful to at least some microscopists is that the final solution should have somewhat the same cloudiness as a gin and tonic, a drink which exhibits an attractive bluish opalescence when not too much ice is present. To a drop of this suspension an equal drop of 4 percent PTA is added, and, after mixing, an aliquot is placed on a grid held by forceps. After allowing adsorption for a few seconds, the excess fluid is withdrawn by a piece of filter paper with a torn edge (Plate 2). The torn edge may seem trivial but better and more delicate contact is achieved with a ragged, torn edge than a smooth cut one. The grid is allowed to dry and is then ready for immediate examination in the microscope.

2. *Rapid Method of Negative Staining.* There are occasions when a very quick examination of a specimen will guide further handling or even yield a diagnosis. The technique described here takes only a few minutes to carry out but it will not yield a

specimen of the same technical standard as the previously described method. In addition, since there is no concentration step, it cannot offer the same sensitivity as the standard method. In spite of this, it is a technique that can frequently provide a diagnosis in the shortest possible space of time. The suspected virus must be in suspension, a drop of which is placed on a grid, held by forceps. After a few seconds have been allowed for adsorption, excess fluid is withdrawn and, before drying has occurred, the grid is washed gently with several drops of distilled water delivered from a Pasteur pipette. Again without drying, the distilled water is replaced by adding a few drops of 2 percent PTA. The final drop is withdrawn by filter paper and the grid allowed to dry. The grid is then ready for examination in the EM and the whole operation should have been carried out in the space of five minutes.

Specific Types of Specimens

Urine. If cloudy or contaminated, urine can be clarified by centrifuging for 15 minutes, at 3000 *g* in a bench centrifuge and then centrifuging the supernatant for one hour at 15,000 *g*. The pellet should be handled as described and stained by the standard method. Since urine is readily available and virus is much diluted, centrifuge as large a volume as possible.

Faeces. Make up a 10 percent suspension of stool in distilled water and clarify in a bench centrifuge. Process the supernatant directly by the rapid method of negative staining or centrifuge approx 2 ml for one hour at 15,000 *g* and stain the pellet by the standard method. Faecal specimens can be examined successfully by the rapid technique, and it appears that only a slight decrease in sensitivity occurs when comparing this rapid technique to the standard method (Plate 3).

Serum. Since serum is very rich in low molecular weight proteins it is necessary to carry out a washing step. Dilute serum with an equal volume of distilled water. The absolute volume employed will depend on the availability of the specimen but 0.2–1.0 ml of serum is a suitable volume. Centrifuge for one hour at 15,000 *g*, discard supernatant, and resuspend pellet, which will not always be visible, to its original volume in distilled water. Recentrifuge as before and, after decanting, process the pellet by the standard method of staining (Plate 4).

Vesicle Fluid. For the best specimen, vesicle fluid should be taken from unbroken lesions. The vesicle is best ruptured with a sterile Pasteur pipette which contains a small amount of distilled water. This water can then be used to irrigate the lesion and give a larger volume of fluid than the vesicle itself could yield. Once transported to the laboratory, the mixture of vesicle fluid and water is applied directly to the microscope grid and staining is carried out by the rapid method. (*Note:* since the viruses in vesicle fluid can be dangerous pathogens, it is important to have a suitable small, well-sealing container for transport) (Plate 5).

Cell Culture. Although a cell culture is not truly a clinical specimen, it frequently happens that the identification of a virus after one pass in tissue culture greatly aids diagnosis. The steps necessary to handle such specimens are as follows. Harvest cells and supernatant together and disrupt the cells, either by several cycles of freezing and thawing or by sonication. Without any clarifying step, centrifuge for one hour at 15,000 *g* and stain by the standard method. The larger pieces of cell debris present do not interfere with the microscopy and frequently are found to contain collections of virus particles at various stages of synthesis (Plate 6).

Allantoic Fluid. As a specimen, this is similar to tissue culture inasmuch as it is

not a direct clinical specimen but represents one growth cycle in the laboratory. Simple centrifugation of the allantoic fluid for one hour at 15,000 *g* followed by the standard method of staining is all that is required for preparation (Plate 7).

Solid Tissue. This will vary from very soft tissue such as brain to very hard specimens such as warts. It is best to prepare these different tissue types by separate techniques.

Soft Tissue. Make a 10 percent suspension of the tissue in distilled water using a glass-teflon Tenbroek-type homogeniser. For very soft tissue such as brain a few strokes of the pestle will suffice while firmer tissue such as liver will need more effort. For reasons of efficiency and safety, scabs from skin lesions are best handled by this technique (Plate 8).

Hard Tissue. Cut the tissue into small fragments and place in a glass or porcelain mortar with a little silver sand. Add a small amount of distilled water and grind with the pestle until the tissue is further fragmented, add more water and continue grinding until a relatively smooth suspension is achieved (Plate 9).

Both types of homogenate can now be handled in the same manner. Clarify for approximately 10 minutes in a bench centrifuge, then centrifuge the supernatant for one hour at 15,000 *g*. Proceed by the technique for standard staining.

Tissue Scrapings (e.g., conjunctival cells). Specimens such as this usually contain very little material and they are best handled by diluting with sufficient distilled water to disrupt any cells present and then staining by the rapid method. It might be remarked here that the electron microscopist is frequently in direct competition with the biologically oriented virologist who is anxious to make an isolate from the same material. When the specimen amount is plentiful, no conflict occurs, as separate samples can be handled individually but a problem arises when only small amounts are available. The most practical solution is to persuade the virologist interested in cell culture techniques to undertake his part of the procedure using sterile precautions and then to hand over the remainder to the microscopist.

Cerebrospinal Fluid (CSF). Dilute with equal parts distilled water and stain by the direct, on the grid, rapid procedure.

Sputum. This is probably the most difficult of specimens for the electron microscopist. It is very difficult to get rid of associated mucous and this results in troublesome background material that can obscure any virus present and reduces the resolution that can be obtained. In spite of this, such specimens must occasionally be handled; the following technique is recommended. Dilute specimen one part to four with phosphate-buffered saline (PBS) and produce as homogeneous a suspension as possible using a Tenbroek-type homogeniser. Clarify the suspension using the bench centrifuge and then pellet virus for one hour at 15,000 *g*. Stain by the standard method.

Immune Electron Microscopy (IEM). All of the previously mentioned specimens can be used for immune electron microscopy by adding specific antisera between the steps of clarification and centrifugation. In some instances, where there is no clarification step, the antiserum is added directly to the crude virus-cell suspension. The most important factor to be considered in producing a specimen for immune electron microscopy is the proportion of virus, or virus antigen, to antibody in the mixture. The final mixture should be in the equivalence range so that reasonably sized complexes will be formed. Too much or too little antibody leads to conditions of antigen or antibody excess with resulting loss of complex formation [6].

Since clinical samples will contain an unknown quantity of virus the antiserum

should be adjusted to a concentration that will give aggregation over the widest range of virus titre that might be encountered. Some judgement of the dilution factor to be used can be derived from the performance of the antiserum in other tests. For example, an antiserum that is active in a complement fixation test to a level of 1:256 will probably be suitable for use in the EM at approximately the same dilution. Theoretically, it would appear that the titre of an antiserum given by a relatively insensitive test such as complement fixation is within the equivalence range of the serum and this will give the best latitude in reactions with varying amounts of antigen.

Since many specimens contain little virus, it is advisable to add the antiserum in as small a volume as possible so that a minimum of dilution occurs. A practical system is to add the antiserum as a tenth the volume of the available virus solution. It therefore is useful to have stocks of antiserum at a dilution of 1:10; addition of this at a tenth the volume of the virus solution gives a final 1:100 concentration of antiserum which is frequently in the range required. Care should be taken that the antiserum is quickly and adequately mixed with the virus suspension so that there is an equal distribution of antibody. The mixture is then allowed to react for a suitable length of time; this can be as short as ½ hour at 37°C or as long as overnight at 4°C. For routine purposes 1–2 hours at room temperature is adequate. After this period of interaction, the mixture is centrifuged as usual for one hour at 15,000 *g* and the pellet processed by the standard method of negative staining. As already stated, IEM makes it easier to visualise viruses with little distinctive substructure (Plates 10–11), and also allows for greater sensitivity as regards low titre specimens (Plate 12). It can also reveal unsuspected antigenic components (Plate 13) associated with the virus [7].

The technique of negative staining for electron microscopy was introduced just twenty years ago, and during these years the method has advanced from a means of obtaining esoteric information about virus structure to an everyday approach to the identification of viruses in clinical specimens. The technique has suffered from the belief that the procedures required for obtaining good micrographs are long and tedious. This article hopes to show that good micrographs depend only on the observance of a small number of important requirements; if these are adhered to, the most unlikely starting material (Plate 14) can provide beautiful micrographs (Plate 15) with a minimum of technical manipulation and in a surprisingly short period of time.

ACKNOWLEDGEMENTS

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PLATE 1. The equipment needed to carry out negative staining. Note that specimens waiting to be examined are contained in a beaker sealed with parafilm and are still inverted. Those already dealt with are in the open beaker.

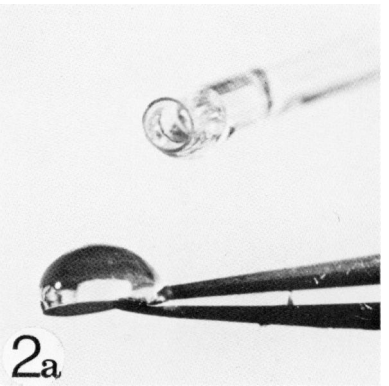

PLATE 2a. In order to obtain a good specimen distribution it is essential that a large drop of virus-phosphotungstate suspension is allowed to adsorb onto the grid.

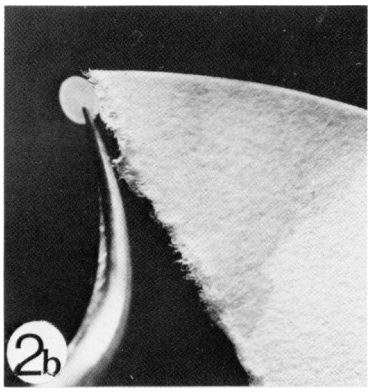

PLATE 2b. After sufficient time for adsorption, the specimen fluid is removed from a grid by means of a torn edge of filter paper. Immediately on drying, the specimen is ready for examination in the EM.

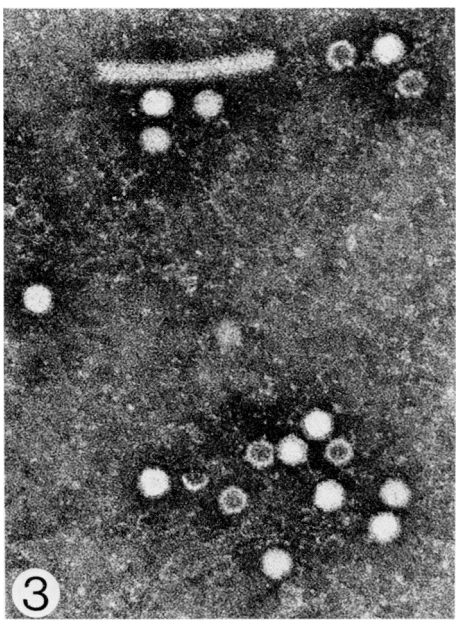

PLATE 3. Faecal samples make surprisingly good EM specimens. They can be processed by either the standard or rapid method of negative staining. The micrograph illustrated here shows an enterovirus that was present in high titre in mouse faeces. The grid was prepared by the rapid technique. × 220,000.

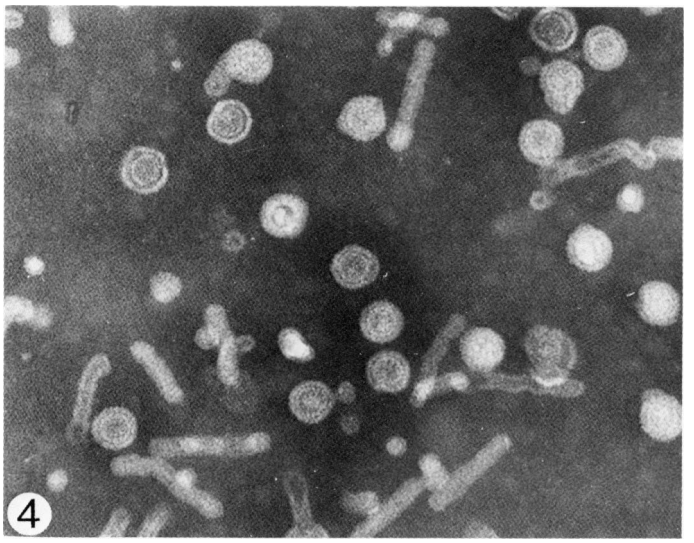

PLATE 4. Sera require a washing step before they are examined since they contain large amounts of low molecular weight proteins. After washing, HBsAg can be seen against an almost structure-free background. × 220,000.

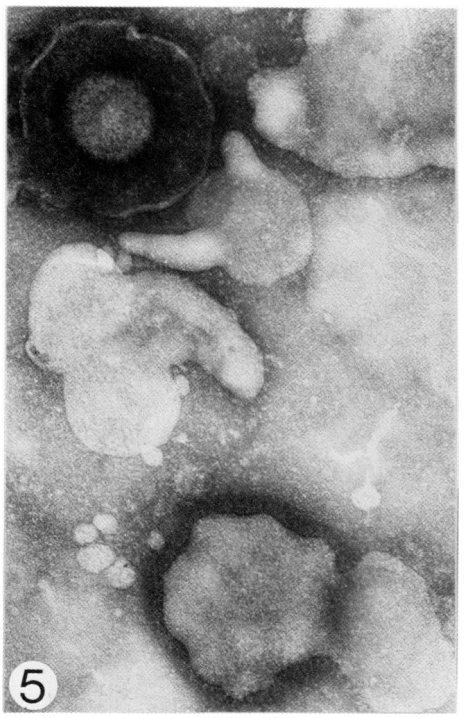

PLATE 5. Vesicle fluid contains a considerable amount of low molecular weight protein. This leads to a loss of contrast but does not prevent visualisation of the virus. This micrograph shows two varicella virus particles, one intact and one broken, in vesicle fluid. × 165,000.

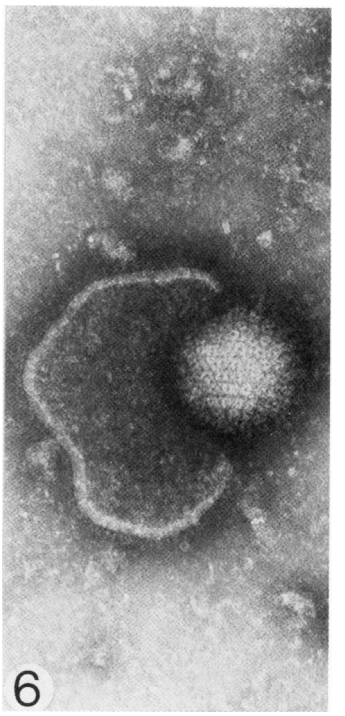

PLATE 6. The method of processing tissue culture cells is simple but can yield preparations allowing good resolution of particles. The icosahedral construction of the inner capsid can be seen in the herpes simplex particle shown here. × 165,000.

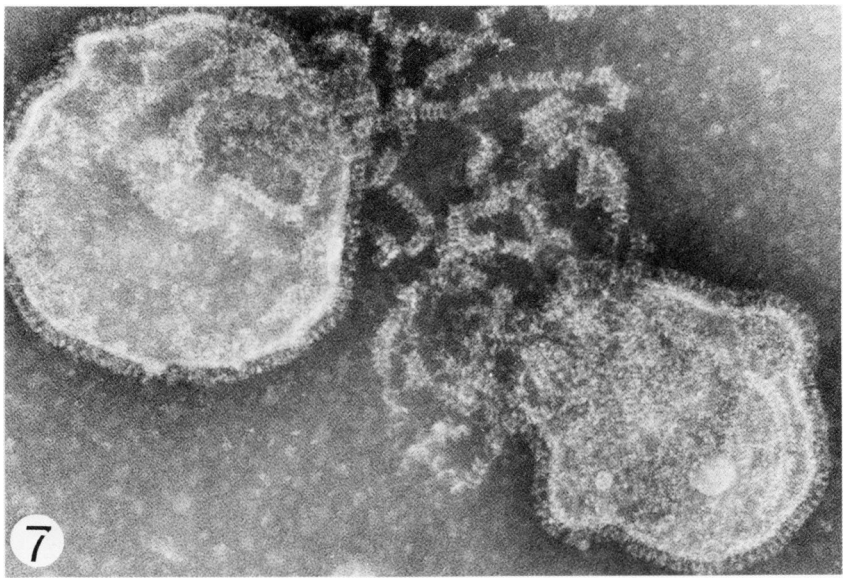

PLATE 7. Two parainfluenza particles from allantoic fluid are illustrated. Both the surface projections and internal helix are clearly resolved. × 165,000.

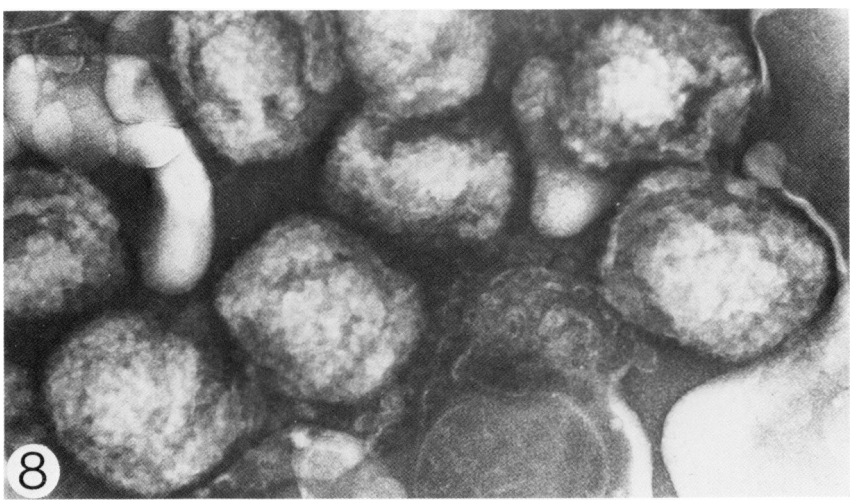

PLATE 8. Smallpox virus is visible among the debris of a scab that has been homogenised. Because of delay in transit there was no viable virus left in this specimen but morphological identification was simple. × 110,000.

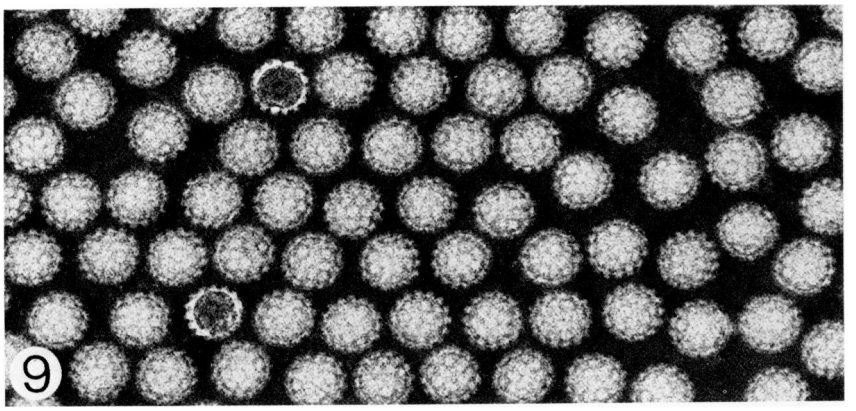

PLATE 9. The human common wart virus is present in lesions to such high titre that a simple extraction procedure with little concentration will yield specimens like the one shown here. The virus is present in pseudocrystalline arrays. × 228,000.

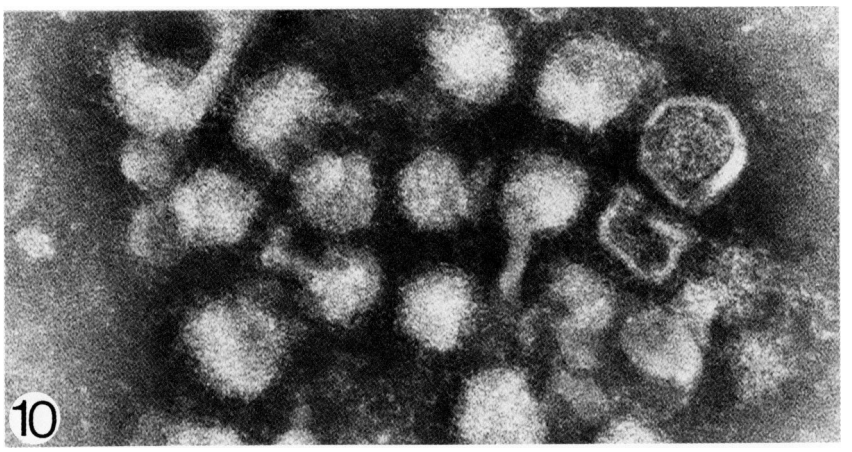

PLATE 10. Viruses having no distinctive morphology are more readily recognised when they are present in the form of immune complexes. This micrograph shows a complex of rubella virus which appears as a poorly defined lipoprotein sac with very fine, inconspicuous surface projections. When viewed only as individual particles it is hard to distinguish this virus from cellular background. × 220,000.

PLATE 13. Immune electron microscopy can reveal unsuspected structures, as shown here. This aggregate contains both complete and fragmented rotavirus capsids set in a background of amorphous material. This amorphous material represents a low molecular weight subunit that is antigenically related to the virus. If IEM had not been used there would be no indication of its presence. × 228,000.

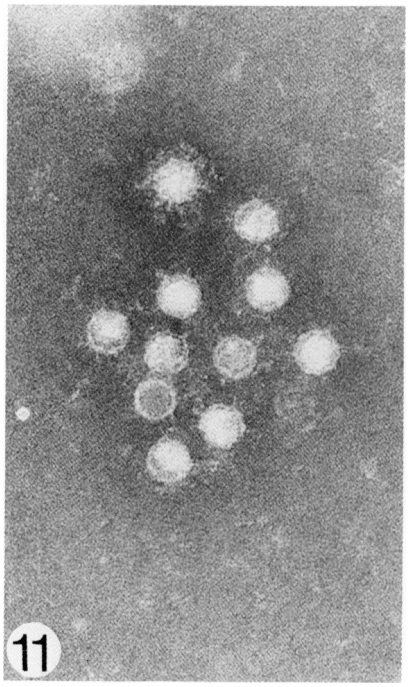

PLATE 11. Addition of antibody makes a small cubic virus more readily recognisable and, in addition, proves its identity through the presence of specific antibody haloes around the particles. The micrograph shows a complex of polio virus and specific antibody. As long as specific reagents are used it is possible to distinguish the three serotypes of polio virus by IEM. × 220,000.

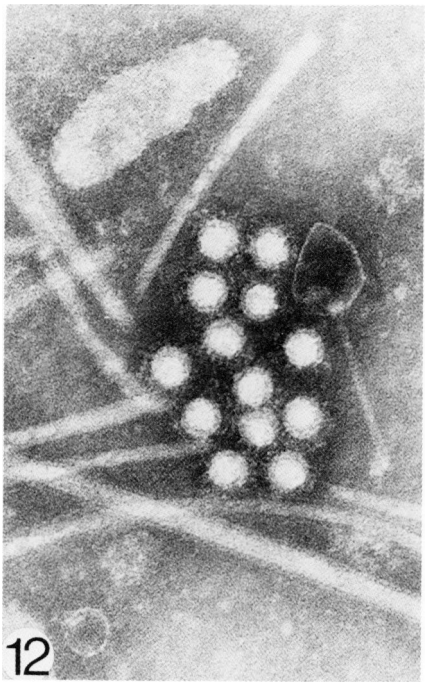

PLATE 12. An immune complex of hepatitis A virus surrounded by the common contaminants found in faecal specimens. Although the virus is present in low titre, complexes stand out from the background material. × 220,000.

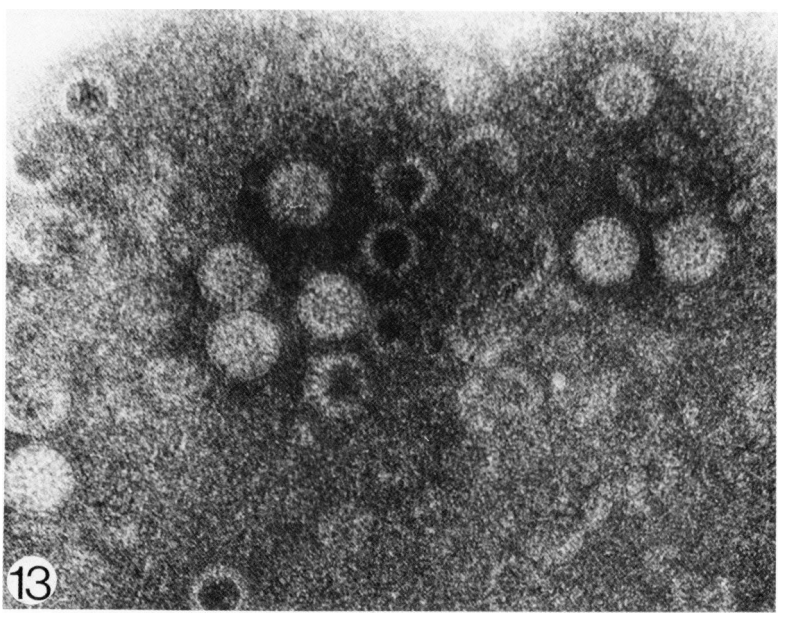

PLATE 14. The starting material for Plate 15.

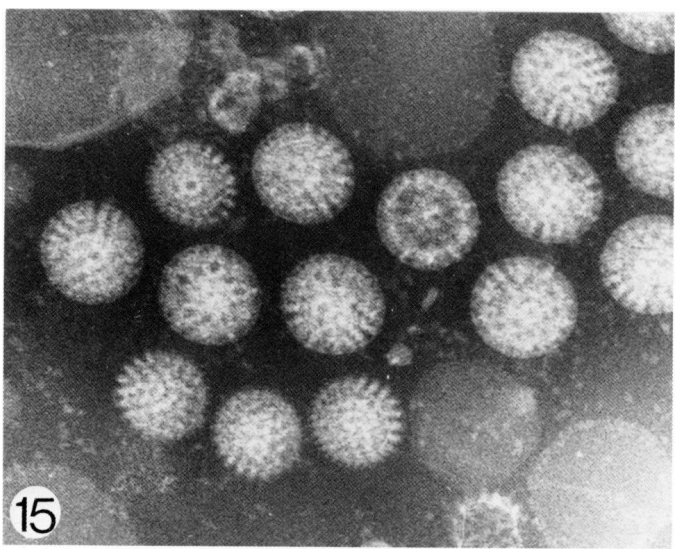

PLATE 15. The beauty that had been hidden in Plate 14. Bovine rotavirus. × 275,000.