

IMES



This is to certify that the

thesis entitled

NEGATIVE CONTRAST ELECTRON MICROSCOPY OF
VETERINARY VIRUSES

presented by

MICHAEL ROCKLAND VERRILLI

has been accepted towards fulfillment
of the requirements for

M.S. degree in MICROBIOLOGY AND
PUBLIC HEALTH

Major professor

Date 2-24-78

P ~~_____~~ 274
~~_____~~ 301
~~_____~~ B
~~_____~~ 3

THES:



This is to certify that the

thesis entitled

NEGATIVE CONTRAST ELECTRON MICROSCOPY OF
VETERINARY VIRUSES

presented by

MICHAEL ROCKLAND VERRILLI

has been accepted towards fulfillment
of the requirements for

M.S. degree in MICROBIOLOGY AND
PUBLIC HEALTH

Major professor

Date 2-24-78

P. 274
~~4-19-30~~ 1930
~~2-1-30~~ 1930
~~1-1-30~~ 1930

L

NEGATIVE CONTRAST ELECTRON MICROSCOPY OF VETERINARY VIRUSES

By

Michael Rockland Verrilli

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1978

9112548

ABSTRACT

NEGATIVE CONTRAST ELECTRON MICROSCOPY OF VETERINARY VIRUSES

By

Michael Rockland Verrilli

Representative viruses of each major morphological virus type were examined by negative contrast electron microscopy (NCEM) according to variables of negative stain type, concentration, and pH, plastic support substrate, and biological wetting agent. One per cent phosphotungstic acid (PTA), pH 6.5, plus Parlodion support films, with or without carbon strengthening, were found to provide suitable negative staining conditions for most viruses studied. Herpes virus particles were optimally contrasted with one per cent PTA, pH 7.0, and membrane-bound viruses could additionally be visualized by one-half per cent ammonium molybdate, pH 7.0, which appeared to give better membrane preservation than seen with PTA contrasting. Butvar support films compared favorably in properties to Formvar supports, and both types of film were judged less preferable to those of Parlodion. Bacitracin afforded a superior spreading agent for negative staining purposes. Established parameters of NCEM were extrapolated to six additional viruses commonly encountered in veterinary medicine, and successfully applied to the examination of distilled water lysates of infected cell monolayers. Methanol precipitation was shown to be an effective concentration procedure for all major morphological virus types.

PLEASE NOTE:

**Figures are very dark copies.
These will not reproduce well
in xerographic copies. Filmed
in the best possible way.**

UNIVERSITY MICROFILMS.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to all who aided with the completion of this study. I would especially like to thank Dr. Gordon R. Carter and Mr. A. Wayne Roberts for their patient guidance throughout this study and their critical suggestions offered during the preparation of this thesis. I am indebted to Mr. Stuart Pankratz for his excellent assistance with the electron microscope and advice on photography, to Dr. Vance Sanger, Dr. Esther Roege, and Patricia Lowrie of the Department of Pathology for introducing me to electron microscopy and for their encouragement, and to Mrs. Betsy Gardner of the College of Osteopathic Medicine for her assistance in the preparation of support films.

I would also like to thank Dr. Maria Patterson for her guidance during a significant portion of my graduate tenure and for assistance in editing the thesis manuscript, Dr. John Dyke of the Department of Pathology, Edward Sparrow Hospital, for his timely help and suggestions, and Dr. Charles Baechler of the Department of Microbiology, Wayne State University School of Medicine, for his advice on negative staining and details about the pseudoreplication technique. Grateful acknowledgement is also made to Paul C. Watkins for assistance in the preparation of viruses, and to Mrs. Dorothy Boettger for her individual efforts in helping to secure the necessary supplies.

This research utilized the Clinical Microbiology Laboratory facilities and was supported in part by a grant from the Michigan Agricultural Experiment Station.

TABLE OF CONTENTS

	Page
REVIEW OF THE LITERATURE	1
A History of Virus Microscopy	1
The Negative Staining Process	8
Advantages and Disadvantages of Negative Staining . . .	11
Desirable Characteristics of Negative Stains.	13
Types of Negative Stains.	14
Ancillary Negative Staining Conditions.	18
Negative Staining Methodology	24
Specimen Support Grids.	29
Support Films	32
Plastic Support Film Production	38
Carbon Support Film Production.	44
Applications of Negative Contrast Electron Microscopy (NCEM)	49
General Biological Applications.	49
General Virological Applications	50
Applications of NCEM to Human Virology	51
Applications of NCEM to Veterinary Virology. . .	56
The Practicality of NCEM as a Means of Viral Diagnosis	59
LITERATURE CITED	63
ARTICLE: NEGATIVE CONTRAST ELECTRON MICROSCOPY OF VETERINARY VIRUSES	72
Summary	73
Introduction.	73
Materials and Methods	74
Results	81
Discussion.	85
Literature Cited.	102

LIST OF TABLES

Table		Page
	ARTICLE	
1	Viruses examined by negative contrast electron microscopy.	80

LIST OF FIGURES

Figure		Page
--------	--	------

(electron micrographs were enlarged to an
optical magnification of 160,000X)

ARTICLE

1	Pseudocowpox particle demonstrating a typical ovoid shape with a complex surface pattern of striated ridges.	89
2	Pseudocowpox particle surrounded by its thin unspiked membrane	89
3	Naked isometric adenovirus virions demonstrating round surface capsomers arranged in icosahedral symmetry. Both complete and incomplete particles are present	91
4	Four paramyxovirus structures are observable by PTA embedding (Figures 4 through 7). Note that all enveloped particles (Figures 4 through 6) possess a well-defined outer envelope covered with a regular fringe of short globular spikes. Figure 4 depicts a whole paramyxovirus virion exhibiting partial negative stain penetration into the envelope interior to reveal the internal nucleocapsid	93
5	Intact enveloped virus demonstrating entirely surface staining.	93
6	Incomplete destruction of the paramyxoviral envelope has occurred, releasing the internal helical nucleoprotein component and allowing entry of negative stain into the envelope to outline the remaining helix.	95
7	Occasional free paramyxovirus helices demonstrating a characteristic width, a central hole, and serrations of regular periodicity.	95
8	Two morphological particle types seen with feline rhinotracheitis virus (Figures 8 and 9). Mature naked virions are composed of elongated hollow prismatic capsomers roughly hexagonal in shape, in icosahedral array	97

Figure		Page
9	Empty capsids of herpesvirus with dark centers and well-delineated outer protein shells are seen. Note side view of capsomer structure on upper particle	97
10	Icosahedral particles with little or no observable surface detail are characteristic of porcine polio-encephalomyelitis virus (picornavirus).	99
11	Pleomorphic equine influenza virus virions showing a well-defined outer envelope which is fringed with long rectangular peplomers. The envelope is resistant to negative stain penetration.	99
12	Bullet-shaped virion of vesicular stomatitis virus with its thin, closely-adherent, finely-spiked envelope .	101
13	Reovirus particle showing its round double-shelled capsid and surface indentations	101

REVIEW OF THE LITERATURE

A History of Virus Microscopy

The first visualization of intact virus particles was probably realized by J. B. Buist at the University of Edinburgh in 1887 (17) while microscopically observing one-quarter micron diameter oval bodies or "micrococci" present in gentian violet-stained film preparations of crude smallpox lesions. Amédeé Borrel in 1904 (12) demonstrated that the larger cytoplasmic Bollinger inclusion bodies found in and separable from fowlpox lesions could be subdivided into smaller infective particles, which he correctly concluded were the elementary nucleocapsids of the etiologic agent. At Hamburg in 1906, Paschen (87) similarly described the "corpuscles" or elementary bodies of vaccinia in carbol fuchsin smears of fresh vesicle fluid.

Woodruff and Goodpasture (1931) successfully correlated the number of fowlpox elementary bodies observed by bright field microscopy to be released from an isolated broken inclusion body with the infectivity titer of the same preparation, substantiating the suspicion that the bodies were those of the virus itself. By increasing the resolving power of the ordinary light microscope through the use of a shorter wave ultraviolet light source and special quartz lenses and condensers, Barnard and Welch in 1936 (8) obtained further convincing dark field ultraviolet photomicrographs of canarypox virus, although smaller viruses could not be seen by this method. Scientific technology through the middle 1930's thus saw little advance in the direct

viewing of virus particles, although it was realized from extensive filtration and analytical centrifugation studies that viruses already isolated and identified (except members of the pox and psittacosis-lymphogranuloma virus groups) were too small to be resolved by the best conventional means, including ultraviolet microscopy.

Significantly, in 1932 Knoll and Ruska (62) constructed the first workable electron microscope, in what was to constitute a landmark achievement in the progression of knowledge about virus morphology. Subsequent substantial improvements in performance by this instrument occurred in the late 1930's and early to middle 1940's, allowing its practical application to the determination of virus structure. By allowing a drop of a simple aqueous suspension of purified virus to dry on a filmed electron microscope grid and observing the outline of the virus particles produced by their back-scattered electrons, Kausche et al. in 1939 (60) first showed photographically that tobacco mosaic virus possessed a small, slender, rodlike form, in agreement with earlier viscosity, ultrafiltration, diffusion, x-ray diffraction, and sedimentation measurements. Shortly thereafter, Stanley and Anderson (1941) similarly established the appearance of several other purified infectious plant viruses to be discretely rodlike (tobacco mosaic, cucumber mosaic) or spherical (tomato bushy stunt, tobacco necrosis) in shape. Other researchers quickly extended this simple method to the observation of various animal viruses, notably the ridged "breadloaf" vaccinia virus seen by Green (1942), the spherical rabbit papilloma (Shope) virus reported by Sharp in 1942 (97), and the large, circular, internally structured influenza virus described by Taylor (1943).

Three major methods designed to achieve appropriate contrast for the electron microscopic viewing of particulate biological material were developed during this period and directly preceded the advent of negative staining (46). Williams and Wyckoff in 1945 (116) first introduced the technique of shadowcasting. Isolated virus particles in suspension were deposited on a support substrate by spraying or from small pipettes, allowed to dry, and a mist of sublimated electron-dense metal (e.g., chromium) cast *in vacuo* at an oblique angle of incidence upon the specimen. The thin layer of deposited metal introduced contrast enhancement by causing differential electron-scattering according to the specimen surface variations, as they trapped different amounts of metal "stain." Furthermore, the metal layer replicated surface detail to an extent, and the shadows cast upon the support surface by the angular deposition were devoid of any metallic deposit, resulting in a true shadow of the specimen particle being studied. The angle of incidence plus the size and form of the shadows produced allowed the three-dimensional determination of the height and contour of the upper particle surface. Although heavy metal shadowing endowed viruses with excellent contrast, drawbacks included laborious preparation, partial and difficult image interpretation, the presence of drying and coating artefacts (e.g., particle distortion and collapse), and coat and support film obscurement of the inner virus structure (53,61,116). The three-dimensional preservation of specimen particles was resolved by Williams in 1953 (117) using freeze-drying, and Anderson (1951) with critical point drying, but the other disadvantages remained.

The study of viruses present within host cells began with the development of techniques for fixation, embedding, and ultrathin

sectioning of tissues in the years 1949 to 1953. As early as 1939 von Ardenne (112) used a steel knife to cut the first wedge-shaped tissue thin sections whose edge was penetrable to the electron beam. Porter et al. (1945) invented a method for growing tissue culture cells on Formvar film inserts placed in roller flasks, fixing them, and examining the thin peripheral portions of the grid-mounted cells for intracellular virus. With this method, Porter and Thompson (1948) were able to photograph thin areas of intact murine adenocarcinoma cells containing mammary tumor virus, and show that such preparations were suitable objects for electron microscopy. Although O'Brien and McKinley (1943) and Fullam and Gessler (1946) unsuccessfully attempted to adapt centrifuges into high-speed microtomes, Pease and Baker in 1948 (88) showed that conventional microtomes could be modified to cut ultrathin sections by inserting a special wedge to reduce the increment of advance, thus rendering sectioning technique a practical method for observing viruses within intact host cells. Concomitantly, Newman et al. (1949) adopted the use of a polymerizable thermoplastic resin, butyl methacrylate, for the hard embedding of tissue culture cells. Combining the ideas of Pease and Baker (88) and Newman et al. (82), Black et al. (1950) and Morgan and Wyckoff (1950) demonstrated tobacco mosaic virus and fowlpox virus, respectively, as discrete structures within the sectioned cytoplasm of infected host cells.

In other important advances in ultramicrotomy, Claude (1948) pioneered the bypass principle for returning the specimen to the cutting position without passing the knife edge and constructed the first knife boats used for floating ultrathin sections away from the knife. Latta and Hartman (1950) introduced the use of very sharp knives made from cheaply available fractured plate glass for ultrathin

sectioning, and in 1956 Fernandez-Moran (32) used the first diamond knife, which facilitated thin sectioning by eliminating the necessity to break and inspect glass knives. In 1953 Porter and Blum (92) developed the first simple practical microtome to be later produced commercially. Palade (1952) investigated the use of osmium tetroxide for preserving fine cellular structure and the effects of different buffer vehicles upon this preservation, as a followup to the studies of Strangeways and Canti in 1927 (102) using dark field microscopy, and Porter et al. (1945) on whole cell mounts.

Gaylord and Melnick (1953) combined the best ideas from these previous approaches to initially describe internal virus detail revealed by thin sections, and Morgan et al. (1954, 1956) gave preliminary reports on the stages of extracellular and intracellular development of herpes simplex, vaccinia, fowlpox, and influenza viruses. Well-fixed and -preserved plastic-embedded pellets of infected tissue culture cells ultramicrotomed and stained by modified sectioning techniques thus provided a powerful tool towards the *in situ* characterization of the structure and biological properties of intra- and extracellular virus. Disadvantages included tedious preparation, and the presence of confusing fixation, embedding, sectioning, and staining artefacts. Virus contrast was also directly related to the uptake of heavy metal stain and to the section thickness (46,53).

Finally, the direct positive staining of viral thin sections and whole particle mounts conferred good contrast upon virus specimens by means of the localized charge attachment of heavy metal ions to selected tissue or cell macromolecular sites, such as proteins or nucleic acids, to increase electron scatter (46); final image contrast depended upon the number of electrons scattered from the stained

particles in depth and the total amount of stain that they absorbed. C. E. Hall initially reported in 1955 (45) that specimens exposed to osmium vapors and other heavy metals during the drying process were impregnated or stained and made more electron-opaque in contrast to their light background. Valentine (1958) first used uranyl acetate to enhance the contrast of the central ribonucleic acid core of unsectioned adenovirus, causing this portion of the virus to become more dense. Huxley and Zubay (1960) used a novel double positive staining method whereby grids with dried virus suspension on their surface were floated first on uranyl acetate, succeeded by lead hydroxide, to cause enhanced staining of one stain by another. Unfortunately, in all cases the anticipated gain in resolvable detail caused by positive staining was not as great as the increase in contrast, and not all virus types reacted equally well with different positive stains, so that positive staining methods were not as applicable to the demonstration of viral ultrastructure as was hoped.

In 1954 Farrant (31) first reported the effects of contrast reversal in electron microscopic images while studying micelle formations of ferritin molecules by positive staining methods. Hall (1955) soon after proposed the future use of electron-dense metals to embed small spherical viruses for high contrast electron microscopic viewing, in place of conventional methods of positive stain impregnation. He encountered, deliberately contrasted, and accurately described virus particles demonstrating an anomalous contrast reversal while examining the electron densitometry of positively stained tomato bushy stunt virus preparations after the virus was exposed to a number of positive stains over a wide pH range. Huxley in 1956 (56) extended

the use of the negative stain concept to tobacco mosaic virus (TMV), demonstrating that TMV is a hollow protein rod from the dark outline of phosphotungstate stain which had penetrated into the central axial hole of its cylindrical nucleocapsid and had surrounded the external protein shell itself.

In a classical and fortuitous investigation, Brenner and Horne (1959) established a simple, exact, routine procedure for the rapid examination of samples containing virus at high electron microscopic magnifications using high contrast negative staining. A neutral solution of electron-dense phosphotungstic acid was mixed with partially purified or pure T_2 bacteriophage and plant virus specimens, and sprayed with a modified vaponephrin nebulizer gun onto grids coated with thin evaporated carbon support films. The phosphotungstate formed a structureless electron-opaque background sheet around and partially over the virus, resulting in a reverse-contrasted final image which delineated the well-preserved fine structure of the virus. Brenner et al. (1959) performed extensive negative staining studies upon the structure of the T-even bacteriophage series and their components by this method. They concluded that current standard chemical and physical preparatory methods (freeze-drying, thin sectioning, positive staining, shadowcasting) were too laborious and limiting for the examination of multiple virus samples, but the newly-established negative staining technique met most requirements admirably. Encouraged by this, Horne et al. (50) then visualized adenovirus particles in this way and showed that the surface of the virion was studded with geometrically arranged subunits. R. C. Valentine (1959) followed with a survey of the negative stain morphology of such proteins as

thyroglobulin, urease, ferritin, L-glutamate dehydrogenase, catalase, and gamma-globulin. Using a combination of phosphotungstate with 0.05% bovine serum albumin added as a wetting agent and latex spheres for reference particles, he was able to correlate observed shapes accurately with known molecular weights and sedimentation and diffusion constants. Huxley and Zubay (57) employed a much simpler and now widely used modification of Brenner and Horne's original procedure. They spread a droplet of a ribosome-phosphotungstate mixture by allowing a thin film of deposited liquid to dry upon a holey carbon support film, and then examined the preparation by inspecting areas of the support film where the ribosome-PTA sheet had dried across holes in the carbon. Good sample spreading and electron microscopic images of increased detail and clarity were obtained.

Major changes occurring since the first years when negative staining was established as a basic ultrastructural technique have been few. Many different materials have been assessed for their suitability as negative stains (52,54,109), and many types of biological specimens examined (39,46,51). New types of support films (47,84) and wetting agents (43) have been tested, as well as the preparatory conditions, including fixing and freeze-drying (81). The original methodology remains proven.

The Negative Staining Process

Negative staining employs the concept of partially embedding electron-translucent specimen particles within a concentrated, dried, structureless electron-dense matrix to produce a contrast enhancement and reversal which is observed in the final apparent electron microscopic image (13,51,52,53,54,84,109).

The basic methodology of negative staining is simple in theory and execution. A small volume of particulate biological material is thoroughly suspended in an equal volume of a dilute solution of a neutralized heavy metal salt. A droplet of this mixture is deposited directly onto a thin, grid-held carbon/plastic support film by mechanical means, a population of stained particles allowed to adsorb securely to the film surface, and the preparation rapidly air-dried at room temperature after filter paper removal of the excess fluid from the grid rim. Subsequently, the specimen is examined directly in the electron microscope (13,46,51,54,55).

Under these conditions, the dissolved electron-opaque salt removes from solution during the final stages of drying to form a thin, smooth, amorphous background film or "glass" of high weight density which surrounds and partially overlies the specimen particles on the support film as a result of surface tension forces (46,61,109). Biological specimens stand out as relatively transparent objects or low-contrast "holes" against this dark background opacity, due to the poor electron-scattering power of their low molecular weight atoms and the buildup of negative stain in an electron-dense, displaced halo (46,50,55,61,84). Peripheral, distal, and proximal specimen substructure and fine detail can be seen as a consequence of the penetration of molecular aggregates of negative stain into the specimen surface features; thin specimen areas are finely and irregularly contrasted because they are rendered more electron-dense, and thicker portions remain electron-lucent (46,55,61).

Structural orientation and detail are largely preserved because the final specimen air and vacuum dehydration on the support film occurs after the stain itself solidifies (46,51,52,58). As the

specimen dries and its water sublimes, a matrix of stain presumably replaces the water rapidly at the interstices and molecular boundaries of the specimen until all previously hydrated regions are filled simultaneously (84). The enveloping stain also progressively hardens as drying proceeds and supports the specimen structure to protect it from surface tension forces so that it cannot greatly flatten, disrupt, or distort. The stain thus rigidly molds the native shape and form of the specimen ultrastructure during the final drying process, and the molecular distortion of the original material is minimized because surface tension forces are dissipated against the stain bed surface only (39,51,52,84).

Since information exists in the observed image only as a result of the particular way in which the specimen modifies the incident illumination by the physical phenomena of absorption, reflection, diffraction, or their combinations, useful electron microscopic images are formed only if electron-matter interactions detectably modify the electron beam. Electron staining is necessary for most specimens of biological interest because of their extreme electron transparency. Negative staining accomplishes electron image enhancement by outlining the sample shape and structure with material of high electron-scattering power. The actual negative stain image formation then primarily involves the process of diffraction, i.e., radiation scattering by the illuminated specimen object, and the collection, or focusing, of the scattered radiation by a suitable electromagnetic optical system to directly produce the final image. Upon passage through the lens, the diffracted radiation is recombined so as to restore precisely in the image plane the actual object phase and amplitude relationships. The final three-dimensional detailed specimen

image recorded is a direct result of focusing variations of radiation intensity (contrast) and scattering produced at the image plane, in proportion to the mass distribution, or the three-dimensional structure, existing in the specimen plane. The intensity and scattering distributions in the image are further represented by the optical density distribution of the micrograph (84).

Advantages and Disadvantages of Negative Staining

The main virtues of negative staining for use in the examination of submicroscopic biological particles lie in the inherent simplicity of its concept, materials, and methodology, plus its rapidity and relative accuracy (39,46,52,55). The speedy development of this technique and its broad appeal have also been furthered by the fact that it allows the direct, easy, high resolution visual impression of particle size, shape, and detail, together with a substantial increase in object contrast (39,46,52). Properly performed, negative staining yields permanent micrographs of ultrathin preparations which show good three-dimensional preservation, high contrast, and fine detail (50,51,54,55). In the absence of denaturation, artefacts, or distortions, the photographic images are consistent in appearance and dimensionally reproducible (84). The ultrastructural detail in the image is easier to interpret than those obtained with other methods (e.g., shadowcasting), and molecular dimensions may be estimated directly from micrographs, provided that the immersion of the specimen molecules or particles in the stain is complete or nearly so. Thus correlative molecular volumes, sizes, and weights based on these parameters can be calculated directly from magnified negative stain images (46,84). Large numbers of freshly-prepared low-volume specimens

or multiple preparations of one sample can be produced and examined quickly; moreover, the technique is applicable to impure or "dirty" specimens (39,50,51,52,84,109). Additionally, negative staining does not require a stain-specimen interaction or temperature extremes, and denaturation of the subject molecules by these causes is minimized (84).

Drawbacks associated with negative staining are fewer, but limiting. They include a certain lack of complete technical precision, because many procedural variables are not entirely controllable, e.g., final stain pH, preparation spreading, differential particle absorption, loss, and recovery, etc. (46,50,61). The use of negative staining technique is restricted primarily to the investigation of isolated particles or their components, in a fluid vehicle, and in high titer (50,51,52,61). Electron microscopic imaging of specimens is limited to only exposed surface views, and cannot reveal internal detail, except when stain fills empty spaces or interiors, or when staining is applied to preparations of particle components or incomplete structures (50,51,109). Complete penetration of negative stain into object structure may preclude useful viewing altogether (109). Induced artefacts can occur as a by-product of air and vacuum drying (liquid flow-induced particle orientation, surface tension damage) and/or staining processes (electrostatic charge disruption) (50,81). Object appearance can depend upon the characteristics of the negative stain used, the specimen type, and the environmental conditions of staining (53,54,81,84). Rarely, specimen images can be difficult to interpret, due to spurious phenomena such as contour effects, phase image or granularity of the negative stain, and two-sided views (39,46,51). Finally, negative staining does reduce slightly the

apparent size of specimen particles, as the stain lies above and below the real edge of specimen structure (51).

Desirable Characteristics of Negative Stains

The requisite properties of a useful negative stain include:

- 1) physicochemical nonreactivity with the specimen being examined, ideally including attendant buffers, salts, fixatives, metal and inorganic ions, cofactors, and prosthetic groups (39,46,51,52,53, 54,66,84,109); 2) physicochemical stability over a wide range of concentration and pH values, plus resistance to electron microscope environmental conditions of drying, heat, and radiation impact (84); 3) high specific electron density or anhydrous weight density, i.e., excellent electron-scattering ability per unit aqueous volume (39,46,84,109); 4) a very good aqueous solubility (39,46,84,109); 5) a high melting point, to obviate running or volatilization under the heat of the electron beam (46,109); 6) extremely small molecular dimensions (Stoke's radius), to allow a large degree of penetration into specimen irregularities and fine detail (46,61,84); 7) relative ease and uniformity of spreading over support film surfaces (53,84); 8) a smooth, uniform, agranular appearance upon solidification into a matrix of constant thickness and density which is free of structural artefacts, e.g., microcrystals, -precipitates, and -deposits (46,51,84).

Valentine and Horne (109) have defined a good negative stain most simply as a compound of high aqueous solubility which produces the greatest difference in weight density or electron-scattering power per unit area between the specimen and the stain itself, and thus causes the greatest contrast enhancement.

The optimal end result of negative staining should be a stained specimen which is well-preserved (no evident structural disruption, flattening, or collapse), contrasty, and maximally and relevantly detailed, as viewed by the observer, and seen in the final photographic image (51).

Types of Negative Stains

Comparatively few materials have been found to be efficacious as negative stains (54,84). The sodium or potassium salts of the complex heteropoly acids derived from phosphate, tungstate, silicate, and molybdate, in various combinations, have been widely used (55, 61,84). The lithium, ammonium, calcium, magnesium, and tris(hydroxymethyl)aminomethane salts are also applicable as heavy metal stains in solution. The uranyl salts of the organic acids, formic, acetic, and oxalic, are less commonly used, as are sodium tungstate, cobaltous tungstate, ammonium molybdate, vanadium molybdate, uranium nitrate, silver nitrate, and cadmium iodide. Methylphosphotungstate, methylamine tungstate, phosphomolybdate, tungstoborate, and uranyl aluminum are more obscure, but applicable (61,84). All usable stains have a density range of approximately 3.8 to 5.7 grams/cm³, all are sufficiently dense to scatter electrons significantly, and all are highly soluble in water and form stain deposits of acceptable uniformity and granularity. Any may be found unsuitable for use with a particular specimen preparation because of reactivity with the specimen and/or its vehicle composition (e.g., buffers, salts, ions, etc.). None exhibits significantly superior properties with respect to staining ability, surface activity, or radiation resistance (84).

The best negative stain to employ for a particular sample is determined empirically by staining native specimen molecules with several different negative stains at different pH's (e.g., 5 to 8) and concentrations (e.g., 0.25 to 4%); one might try different staining procedures, different wetting agents, and various methods of preservation, such as prior fixation or freeze-drying (61,84). It should be kept in mind that the detail visible in the specimen depends upon the degree of penetration of specimen regions by the negative stain molecules; thus, using heavy metal stains at pH's and concentrations yielding maximum contrast or the most aesthetically pleasing image will not necessarily insure the presentation of the finest detail (54). In any case, specimen morphology can be greatly influenced by varying the stain characteristics and the conditions of staining to produce the best visual impression in terms of relevancy and informational content (39,53,81).

Phospho- and silicotungstate stains are among the most popular of the complex heteropoly acid heavy metal salts. The sodium or potassium salts are the most commonly prepared forms and currently afford the best general purpose negative stains (84). Generally described, they are anionic (negatively charged) stains consisting of a heteropoly acid with 12 to 24 tungstate residues coupled with a simple residue of phosphorus (47). These stains are best used as negative stains for specimen particles and macromolecules above their isoelectric point; they tend to positively stain at low pH values and are usually employed at pH's greater than 4 (46,84). Titrating phospho- or silicotungstic acids to a near-neutral pH with normal NaOH or KOH results in the respective stain. Practical aqueous concentrations range from 0.5 to 2% and pH values from 5 to 9 (46).

These stains possess high contrast and fine granularity, preserve detail well, and project a realistic three-dimensional appearance in the final image (13,46). They are highly soluble and stable over long periods of time, when pH changes due to CO_2 dissolution are prevented. Relatively high concentrations of nonvolatile salts (especially phosphate buffers) can be tolerated without detrimental effects (46). Disadvantages include the more frequent occurrence of structural flattening, disruption, or collapse, reaction with certain systems (e.g., protein buffers), undue stain concentration around the specimen, and poor three-dimensional preservation of very large biological structures (13,46). Electrostatic stress at the lipid-water interface of membranes due to the adsorption of stain polyanions to surface cation groups can cause damage or artefact formation (39,52,54).

The uranyl staining compounds consist of uranyl ions or complex ions containing uranyl groups. Their high atomic weight atoms endow them with the greatest electron-scattering potential of all heavy metal stains. Uranyl stains react avidly with proteins and particularly nucleic acids through electrostatic and coordination complex interactions with specimen carboxyl and phosphoryl groups (47). They generally afford higher contrast than other negative stains, including the complex heteropoly salts. Their smaller molecular size allows greater penetrability of specimen structure (84). These compounds afford excellent contrast media for small or periodic structures, and for outlining nucleic acids. Concentrations used range from 0.25 to 15%, and pH's between 4 and 5 (46,84,94). Uranyl acetate is the prototype of the uranyl heavy metal salts. It is especially recommended for the negative staining of specimens below their isoelectric point.

Highly contrasted fine details over a wide range of specimen concentrations is a distinct advantage (46). A number of disadvantages associated with this and other uranyl stains contribute to their lesser applicability in comparison with the tungstate derivatives. Insoluble precipitates are formed with many proteins and buffer salts, or are present in the stain itself, yielding grainy, grossly speckled or splotched backgrounds of uncontrollable contrast in unclean samples (94). Stain spreading is less than optimal and results in patchy backgrounds due to nonuniform wetting of the support surface. Good preservation of specimen structure is usually seen, but softer particles tend to shrink slightly or distort (13). The size of the granular background deposition is greater, and a much less dense collar of contrasting stain forms around the specimen. Although the stain possesses some cross-linking properties and tends to partially fix many specimens and their components (especially protein and lipoprotein complexes), it can induce artefacts and pseudocontrasting (54). It is sensitive to photochemical change and unstable to pH's greater than 6, in the absence of chelating agent, so that it is usually applied in dim light or dark below this pH (46,61,84). The two-dimensional flatness of its recorded image may also detract from its appeal (46).

Ammonium molybdate has been proven to be advantageous as a negative stain in certain instances. It has been shown to be a markedly superior stain for the intact preservation (without fixation) of osmotically-sensitive specimen structures, including labile membrane-bound systems, organelles, and particles (e.g., microsomes, ribosomes, mitochondria, erythrocyte membranes). Its unique applicability is related to the fact that the tonicity of the stain is adjustable over

a substantial range of concentrations to match the requirements for stabilizing a particular membrane structure (46,78). Good preservation is a result of the high osmotic pressure that the stain exerts and the probable attendant favorable surface charge of the stain ions; structural stability is comparable to that of material processed for ultrathin sectioning (78). Predominantly one-sided specimen images have also been obtained by ammonium molybdate embedding (46, 53). Disadvantages of the stain lie in the lesser amount of detail observed and a softer contrast. The compound consists of two ammonium ions coupled with a molybdate residue. Practical concentrations range from 0.5 to 2% and pH values from 6.8 to 7.4 (46).

Ancillary Negative Staining Conditions

Additional conditions necessary for good negative staining results have been described (39,51,53,55,84,94). Optimum circumstances for the preservation of specimen material in liquid suspension, for spreading, drying, contrast, mounting, and microscopy should be established, because a negative stain with properties suitable for a particular specimen in a certain environment may be unsuitable for a different type of specimen (53). Ideally, it is desirable to obtain an optimally thin stain bed of uniform thickness and homogeneity, and just sufficiently deep to engulf entire specimen molecules or particles (53,84). Moreover, the objects of interest should be well separated in this thin film of negative stain (53,94). Since the resolution of molecular features is realized only if the negative stain is deposited immediately at the stain-specimen boundary to produce a maximum density contrast, nonuniform spreading and/or the deposition of material with a low solubility/volatility or with a weight density

less than that of the negative stain employed should be restricted (61,84).

Prolonged exposure of specimen material to the negative stain should be avoided, as contact of long duration between the stain and the specimen before the final drying process occurs can result in the induction of structural and/or chemical alterations in the material being examined. Staining times may vary empirically from several seconds to several minutes (53,55).

The pH of the mixture of specimen and stain must be maintained to insure specimen preservation, and small amounts of volatile buffer can be used to accomplish this. Near-neutral pH values have been shown to provide this, plus minimal background structure and maximum specimen detail (39,51).

Excessive amounts (greater than 2 mM) of buffer salts, inorganic proteins, cofactors, ions, prosthetic groups, coenzymes, or other non-volatile substances (e.g., carbohydrates, substrates, reducing agents, detergents, etc.) in the specimen should be avoided (84), and if present in bulk, should be removed by rinsing the grid preparation several times with distilled water or a volatile buffer, such as 2% ammonium acetate or ammonium bicarbonate at neutral pH, prior to negative stain application (51,53,55). Undesired substances may be removed by agar filtration (81), by allowing dialysis through a support film into an agar base (4), by dialyzing against a volatile buffer (51,53,54,55), or by elution upon buffer-soaked filter paper strips (54,113). The specimen can be centrifuged and the pellet gently washed free of contaminants and resuspended in distilled water or volatile buffer (51,52), or gel filtration on 2 to 4% agarose gel columns with a water eluant applied to the sample (94). Any buffers

used during negative staining should be present in low concentration and free from material that is likely to crystallize or form a precipitate upon drying (39,51,52,53).

The effects of specimen contaminants upon the final result of negative staining are manifold. The presence of undue amounts of salts (especially from density gradient samples, cultural media, buffers) often causes the formation of small crystallites or "trees", patchy, mottled stain backgrounds or coarse granulation of the stain itself which obscures the object of interest and/or causes grossly misleading artefacts and aesthetically unappealing images (53,54,94). Certain ionic compounds such as sodium chloride and ammonium sulfate are readily volatilized by the heat of the electron beam and during their degradation cause stain migration which obliterates the specimen image (84). Serum, spreading base proteins, or those present in body fluids or culture media may act as surfactants, causing uncontrollable stain spreading and/or increased background granularity, with poor contrast results (94). High concentrations of carbohydrates (e.g., sucrose) may "glaze" the specimen and obscure detail. The presence of reagents such as aldehyde fixatives or some types of proteins can reduce the wettability of support film surfaces (84).

Spreading properties of negative stain preparations depend upon the concentration and volume of negative stain in relation to the concentration, size, shape, and molecular weight of the specimen (39, 52,53,84). The proportions of specimen and negative stain in a preparation thus can be critical and should be adjusted to attain a uniform distribution of stain and specimen material (54,55). Suspensions containing a relatively large amount of stain in proportion to specimen material (e.g., protein concentrations less than 10 μg per

ml) will often result in the formation of large, thick, electron-dense globular pools in which particulate material in small numbers is deeply embedded and obscured (13,51,52,53,54). Conversely, highly concentrated specimen in the presence of small amounts of negative stain (e.g., protein concentrations greater than 100 μg per ml) can give large, amorphous aggregates of electron-transparent specimen particles that are poorly distributed, preserved and/or stained. These appear interspersed with small, dark, poorly spread and widely separated stain droplets without specimen upon the support film, and areas of support film barren of negative stain layer (51,52,53,54). Plastic and/or carbon support substrates affect the spreading properties of negative stain preparations due to their hydrophobic or hydrophilic properties, age, and moisture content, and these factors should be taken into consideration in adjusting stain-specimen ratios (13,53,54,84).

Extremely small amounts of low molecular weight organic or inorganic surface-active compounds can be added to aqueous particulate negative stain preparations (54). These facilitate reproducible spreading of preparations upon support films in the absence of trace soluble proteins or surfactant impurities that can normally be found in some biological specimens, or in the presence of support films exhibiting poor wettability (55). Spreading is important because the amount of spreading affects the stain bed thickness and the image quality; too much spreading reduces contrast, and too little spreading causes object obscurement (43,84). Spreading agents enhance and control preparation spreading by lowering the surface tension of the staining solution and reducing the contact angle of liquid and solid surfaces; thus, the area of support film surface wet by droplets of a

given volume of negative stain increases, and a thinner stain bed results (43). Disadvantages include the fact that the wetting agent may react with the specimen surface protein, and the presence of low molecular weight particles of wetting agent can obscure very small structural details at very high resolution (43,54). Gregory and Pirie (43) have stressed a number of important characteristics for a biological wetting agent to possess: 1) it must have extremely small, nonvisualizable molecules; 2) it must be compatible with most commonly-used negative stains; 3) it must be biologically acceptable (nonreactive) for a wide range of specimen types; 4) it must not readily recrystallize or precipitate upon drying; 5) it must not form visible micelles which obscure the specimen or its detail; and 6) it should be commercially available.

The spreading agent most commonly used in conjunction with plastic supports is bovine serum albumin (BSA; Cohn's Fraction V), in final concentrations ranging from 0.005 to 0.05%, w/v (39,51,52,53, 55,94). Although BSA easily causes uniform stain spreading, its disadvantages include ease of denaturation and flocculation, plus an increased stain background granularity and obscurement of fine detail, due to its molecular size. It also tends to aggregate in the presence of some negative stains (55,94). Sucrose crystals (0.4% or 0.08 to 0.1 M) may also be added to the negative staining solution (39,53,55). A more uniform layer of stain results upon drying than that seen when BSA is used, and added sucrose can reduce the damaging effect of a hypotonic negative staining solution upon osmotically-sensitive structures (39). However, specimen contrast and detail can be reduced as a result of coating (55). Solutions of octadecanol in hexane have been used to form a monolayer over droplets of aqueous specimen suspension,

causing the wetting of the support film in the final stages of drying so that stain dispersion occurs (41). Wetting probably occurs because excess liquid is drawn away beneath the monolayer upon blotting to leave the latter upon a residual film of liquid. However, the technique requires the use of specially prepared carbon films and is limited to specimens that are not sprayed onto grids (41,43). Gregory and Pirie (43) have suggested that the cyclic polypeptide, bacitracin, is superior to BSA because it is a small nonresolvable protein which meets established criteria and causes better spreading at one-tenth of the concentration required for BSA. A smaller meniscus of negative stain surrounding the specimen and greater negative stain penetration of specimen structure are also distinct advantages associated with using bacitracin as a wetting agent. Horne (52) has employed glycerol or propylene glycol for certain specimen types. Oliver (84) suggests the use of methylphosphotungstates and methylamine tungstates, heteropoly complexes of the methyl esters of phosphoric acid and methylamine, respectively, with tungstic acid. Although less valuable as negative stains, these compounds are compatible with salts of phosphotungstic acid. When added in small amounts, they are useful because of their great tendency to wet the support film surface and to spread sufficiently to deposit large areas of thin uniform stain bed. The enhancement of negative stain spreading upon carbon support films has been accomplished by using carbon tetrachloride treatment (57), high-voltage glow discharge cleaning (47,55,61,93), detergent (73), and ultraviolet light irradiation in short doses (55,61).

Negative Staining Methodology

The simplest and probably most widely used method for embedding aqueous biological specimens within negative stain remains the drop method (13,39,46,54,57,61). Plastic and/or carbon-coated grids are picked up by the extreme edge, clamped with fine-point forceps and the forceps placed upon a flat surface with the support side of the grid facing upwards. Using a clean Pasteur or micropipette, a small droplet of specimen (2 to 5 μ l) suitably diluted in distilled water or volatile buffer is placed upon the grid surface in a bead barely extending from one edge of the grid to the other. The drop is sized so that the fluid is completely retained upon the film surface by surface tension forces, and care is taken not to break the support film by avoiding contact between the grid and the delivery device. After allowing a suitable length of time for the absorption of specimen particles to the film, most of the drop is drawn away at the grid edge with a piece of cut adsorbant paper. Immediately following the removal of excess fluid, an equivalent drop of rinse or negative stain solution is applied before the film surface can dry (46,84). An appropriate rinsing solution is used before negative staining if the specimen contains a high concentration of buffer salts, reagents, etc., that will interfere with stain deposition. If the negative stain solution itself constitutes a suitable rinse, rinsing and staining occur simultaneously (84). The end step is the application of a stain droplet to the grid and the removal of excess to leave a thin aqueous film consisting of a monolayer of negative stain and specimen particles upon the grid surface (46,55,84). Thorough drying in the air or over a desiccant is allowed to take place before examination, so that derangement of the stain deposit (e.g., boiling) won't result

from the rapid vaporization of stain occurring if the specimen is suddenly placed in a high vacuum when incompletely dry (84). A time series for specimen-film and specimen-stain contact can be run to determine the optimal time period for particle distribution and staining, and a dilution series with distilled water or volatile buffer made to find an optimal specimen concentration for negative staining (46,61). Alternatively, the specimen and negative stain may be mixed together in different ratios, allowed to contact for a specific time period, and applied to the grid film as a drop in a single step (46,54). This variation is particularly useful for highly purified specimens, stain-unstable specimens, or cases where specimen particles are so poorly adsorbed by the support film that the application of rinsing and negative staining solutions removes the specimen. Specimen particles seen when the drop method is employed are those physically attached to the support film, plus those remaining after residual fluid is removed from the grid (46). The advantages of drop staining are that it produces excellent results with minimal time and effort, and it can be used with a wide variety of specimen types, including those containing large amounts of nonvolatile material, as deposition of material other than stain and specimen in the stain bed is avoidable (61,84). Other advantages include the selective adsorption of only specimen and stain, and the use of low titer specimens of less than 10^6 particles per ml (13,46,53,61,81,84). Disadvantages include frequent selective aggregation of specimen particles (13,46,53,81), the presence of orientation artefacts due to liquid-flow induced reorientation of particles (81), the occurrence of specimen damage (distortion and collapse) due to surface tension forces (53,81), and the dependency of obtaining thin well-spread preparations

upon conditions of film wetting, the quantity of specimen adsorbed, and the stain properties (84). This signifies that the proportion of specimen particles by size, appearance, or other criteria cannot be studied with this method (61).

Flotation or "successive" methods are variations of the drop method and offer the same disadvantages and advantages (46,84). Specimen particles are applied to the surface of a filmed grid either as a drop or by gently lowering and floating the grid upside down upon a small volume of sample resting upon a flat nonwetting surface (e.g., Teflon, Parafilm, dental wax, or a spot plate depression). After an appropriate time period has elapsed allowing particle attachment to the grid surface under the environmental conditions of the applied solvent, the grid is successively transferred to float upon small puddles of rinsing or staining solutions (46). To avoid exposure of the specimen to surface tension forces during specimen adsorption, rinsing, and staining, the grid can be floated upon the specimen; subsequent washing with suitable rinse and negative staining are performed underneath the grid by rapidly drawing away one solution from beneath the grid with a capillary pipette while simultaneously adding the next solution from the other side with another pipette (61,81). In all cases the grid is finally removed from a negative stain surface, drained of excess fluid, and dried. The final specimen particles seen are those which are physically immobilized upon the support film by favorable collisions. Stain molecules diffuse to the film surface and unattached specimen particles are released into a large stain volume rather than remaining near the grid, so that untoward effects of solvent change are minimized. This allows the study of environmental perturbations occurring with

refractory solvent systems, such as high concentrations of urea or ammonium sulfate (46).

A low pressure glass vaponephrin nebulizer can be used to generate a mist of small, fine droplets (5 to 20 μ in diameter) from a particulate specimen suspended in stain plus distilled water or volatile buffer, and these droplets can be directed onto the surface of a grid support film (46,61). Fine droplets are produced by using air pressure from a hand bulb to drive the fluid suspension in a sealed chamber against a diffuser bulb or glass bead and out through a nozzle held 2 to 3 cm from the grid (54,55,61,94). The best size droplets are those giving discrete spread areas slightly less than the opening of a 200 mesh grid, and these can be obtained by adjusting the grid-to-muzzle distance (55,94). Consistent, uniformly thin deposits of negatively stained specimen naturally result if a wettable support surface is used and if the sprayed solution itself has good ability to wet and spread over the surface. The spreading ability of the solution and the film cause the droplets to spread to the limit permitted by surface tension and volume to give a bed of uniform depth and constant stain mass deposited per unit area of support film, except at the edges (53,84). The preferential adherence of certain types of particles or contaminants over others to the film surface cannot occur with this method, and particles of different size or adsorbability are seen (46,61). Specimen particles tend to separate well from each other so that all particles contained within a known volume can be directly quantitated if an internal reference standard is run (13,52,53,55,61,94). Small volumes of specimen are required, but large numbers of grids can be simultaneously prepared. The droplets produced are small enough so that they dry rapidly and

surface tension and drying effects upon specimen structure are reduced (53). The principal disadvantage of the spray method derives from the need to restrict severely the amount and types of substances present in the sprayed solution other than stain and specimen; material of limited solubility and low density can deposit around the specimen particles causing a reduction in density contrast or stain derangement by their breakdown, resulting in a loss of fine detail (61,84).

Because the drying of the aqueous film proceeds from its edges, the central area covered by the droplet is the last to dry and tends to contain the highest concentrations of such minor solutes, which are held in solution until the final stages of drying (84). Other disadvantages associated with the spray method include requirements for special spraying apparatus and its thorough cleaning after each use (84,94), safeguards against aerosolization when pathogenic specimens are prepared (61), and the need for high specimen titers, usually greater than 10^9 particles per ml (13,55).

The concept behind filtration methods of negative staining involves placing a drop of aqueous specimen upon a finely porous surface and allowing the fluid to diffuse away and leave the specimen particles behind, to be later attached to a support film (61). Pseudoreplication technique employs agar for the purpose of filtration (55, 81). One or more drops of purified virus suspension are individually placed upon the flat surface of a small 1.5% agar block, spread with a clean glass rod or pipette, and allowed to semi-dry. The preparation is covered with a drop of support film solution, drained on edge, dried, the block trimmed, and the plastic film with the specimen particles semi-embedded in it floated onto the surface of the desired negative stain. After a suitable length of time, the preparation is picked up,

drained of excess stain, and dried. The paper filtration method is a simpler modification of the pseudoreplication technique which uses a vacuum filtration through filter paper (81). The plastic support film is picked up from a water surface with filter paper, semi-dried, and cut into small squares. A square is placed upon a filter holder, a drop of specimen suspension pipetted onto the middle of the square, and suction applied. Subsequently, the square is cut into pieces slightly larger than a grid, and the film stripped onto the desired negative stain. Carbon coating can be applied as a final step for both procedures. The advantages of using filtration methods are that they allow the examination of specimens containing certain types of undesirable material (e.g., density gradient salts) and the specimen, if fairly pure, can be easily concentrated by overlaying drop upon drop of sample within a prescribed area. Also, more even, unaggregated preparations result, less negative stain coalesces around the specimen, and orientation artefacts do not occur. Disadvantages include incomplete trapping of certain particles or their structural elements by the support film from the filtration surface, the embedding of contaminants from agar with the specimen in the support film, and the lack of complete protection against particle distortion or collapse. Negative staining is paler or can turn positive. Occasional particle overlap can occur (61,81).

Specimen Support Grids

Specimen support grids are small, thin (50 μm to 0.3 mm), disc-shaped metal screens that are designed to hold thin sections and/or support films by means of a square array of bars for relatively unobstructed viewing in the electron microscope (14,25,40).

Grid size varies, depending upon the electron microscope and the grid holder used, with a standard diameter of 3 mm O.D. being used in the Philips, Hitachi, Zeiss, and RCA microscopes, and 2.3 mm O.D. being suitable for Siemens equipment (14,25,40).

Similarly, grid compositions may vary. Metal grids of copper, silver, nickel, aluminum, tungsten, gold, titanium, stainless steel, rhodium, molybdenum, beryllium, platinum, and palladium are commercially available. Nonmagnetic copper, nickel, and gold grids are most commonly used, with copper representing the least expensive and most popular of the three. Copper grids are usually chosen except where chemical and catalytic treatments or temperature extremes may affect them, in which case nickel, gold, stainless steel, rhodium, or platinum types are usually substituted. Carbon or nylon mesh grids may be used where metal grids of any type are undesirable (14,25,40).

Grid designs range from simple coarse screens to elaborate asymmetric or finder grids with letters or numbers incorporated into the mesh composition to enable the easy location of specific grid squares for repeat viewing. Other grid types are designed for special purposes, e.g., the use of tabbed grids for picking up extended nucleic acid strands from protein monolayers (the Kleinschmidt technique), special slot hole/bar (Sjostrand) grids made for the viewing of entire serial sections, or "honeycomb" hexagonal array grids for extra support (14,47).

Specific mesh size or wire spacing series (50, 75, 100, 200, 300, 400, 500 lines or bars per inch) are manufactured, with 100 to 400 mesh most commonly used. The designated sizing refers both to the area of the specimen which is self-supporting between adjacent grid bars, and the percentage of open grid area per linear inch, and hence

the proportion of ideal specimen area that it is possible to examine. Thus the more open the mesh, the smaller the proportion of specimen that is obscured by the grid bars: 50 mesh grids contain about 95% open area, while 400 mesh grids have about 35% (40). Grid squares may also vary from large open single holes for viewing whole large sections without the obstruction of the grid curves to exceedingly fine screens (1000 mesh) for the examination of particulate specimens with maximum support (47).

Grids are prepared from sheets of fine electroformed or woven metal screening by means of a screen punch of selected diameter, or by photoengraving, i.e., etching each individual grid onto photographically reduced patterns (Athene type grid). Punched grids are inexpensive (about one-quarter the cost of the photoengraved type), but they tend to be rough-edged or to exhibit surface warp as a result of the punching process; uneven mesh can occur as a by-product of the process of electrolytic metal deposition used to form the mesh sheets. Photoengraved grids are smooth on both sides, completely flat, bordered, contain more open area, and are preferable to punched grids, despite the extra cost (25,40).

Three or four hundred mesh grids are best for negative staining work with bacteria, viruses, macromolecules, or other extremely small biological particles (55). Although the advantage of large open viewing areas is sacrificed, the increased number of grid bars per inch offer both greater support of thin plastic support film per unit area, and more metal surface to aid in absorbing and conducting the heat of the electron beam away from the specimen and the support film to the specimen cartridge holder, so that greater specimen stability results

(40). The use of round instead of square grid holes also avoids the point of support film strain occurring at corners (25).

Support Films

The low penetrating power of electrons in the 50 to 100 kV range requires that small biological objects such as bacteria and viruses be mounted upon thin (2 to 20 nm) continuous grid-held support films for examination in the electron microscope (14); many biological specimens are also too thin or too small to support themselves across the larger openings of a grid square (14,40,53). Support films are thus constructed for two major purposes in negative staining work: 1) to mechanically position and hold the specimen within the electron microscope for examination, and 2) to provide a suitable surface over which stain and specimen are distributed in thin layers. In addition to negative staining purposes, support films may also be used for the examination of sections of water-miscible embedding media, for the study of large or serial sections placed on slot/bar grids, for astigmatism correction during routine instrumental alignment procedures, and for the study of surface topographies formed by replication techniques (47).

Hayat (47) and Oliver (84) have pointed out the most important attributes for support film material to possess. These characteristics include: 1) High electron beam transparency, in order to minimize elastic and inelastic scattering or background "noise" of the electron beam (less than 5% absorption) so that image quality is preserved. A suitable material possesses atoms of low atomic number and density which imbue the cast film with minimum intrinsic structure. 2) Adequate strength and mechanical stability under manipulations of the

preparative procedure and the impact of the electron beam, so as to give a clear, artefact-free image through rigid support of the specimen. Exceptionally strong films can be made extremely thin, yet minimal mechanical/thermal drift and shrinkage occur. 3) Topographically, the films should be free of dust or dirt particles, ripples, wrinkles, folds, troughs, crests, rifts, pits, holes, or other inhomogeneities. They should be smooth, and of even, continuous thickness. 4) The film surface should be receptive to aqueous media, i.e., it should have excellent and uniform spreading qualities which enhance the tendency of the negative stain and the specimen to deposit in an acceptably thin, artefact-free bed. 5) Supports should exhibit stability in a high vacuum and resist chemical solvents and extremes of temperature. As such, films constitute a necessary evil: they add nothing to specimen resolution or contrast, yet they generate image noise or artefacts which degrade the final photographic quality (13,84).

Potentially useful thin specimen support films can be prepared from many different types of material. Organic polymers such as Parlodion (collodion, colloidin, or nitrocellulose) and Formvar (polyvinyl formaldehyde) are commonly used, as is evaporated carbon (14,47,84). Butvar resin and other plastics (perspex, cellulose acetate, cellulose acetobutyrate, Bedacryl 122X, polyvinyl alcohol), nylon, glass, uranyl nitrate, graphite oxide, mica, beryllium or beryllium/aluminum alloy, aluminum oxide, alumina, silicon, silicon carbide, silicon monoxide (14,84), and chemically or thermally exfoliated crystals (e.g., vermiculite, molybdenate) have also been employed (44). Recently, Hahn and Baumeister (44) have suggested that synthetic polymers such as atactic and isotactic polystyrene or poly(N-vinyl) carbazole are superior to conventional materials due

to the properties afforded by inherent aromatic stabilization. In any case, the choice of material rests upon the best combination of desired inherent film properties (density, stability, chemical nature, internal and surface structure, etc.), ease of fabrication, nature of specimen, and grid type, with the former two characteristics being the most important (84).

Plastic support films enjoy widespread popularity because they are easily and reliably produced, plus their excellent electron transparency and uniformity depends only upon geometrical thickness, i.e., suitable preparation technique (84). Plastic surfaces are also more hydrophilic and thus allow more uniform spreading of negative stain preparations (46). Major drawbacks associated with such films are that they undergo substantial degradation (40 to 80% mass loss) during electron irradiation, they exhibit a persistent tendency to shrink and drift upon first beam contact, and they cannot be made thinner than about 20 nm (14,40,47). Aging or exposure to heat, light, and humidity causes plastic films to become less beam stable, less wettable, patterned, holey, and/or more brittle (47,67,84). Plastic films are used mostly for routine medium to high resolution work, with a magnification limit of 80,000 to 100,000X (14,61).

Parlodion affords a clean, relatively smooth, uniform substrate which possesses a very fine-grained, amorphous structure (47). Its relative hydrophilicity and high avidity for proteins facilitates the spreading of particulate aqueous solutions into unaggregated thin films. Difficulty of preparation is minimal (84). Additionally, it tends to sublime (65 to 85% mass loss) under the electron beam, which preserves fine specimen details (44,94), but simultaneously causes faster instrumental contamination rates (84). Other disadvantages

include a comparative lack of mechanical strength (compared to Formvar and Butvar), with a tendency to disrupt as a result of surface electrostatic charge buildup under the electron beam, especially when films of less than 200 nm are used (47,84). Although it is stable in a solution of ethyl or amyl acetate or acetone when refrigerated in a dry, dark place, exposure of Parlodion to light and air or aging causes its decomposition into insoluble contaminants, chiefly free acetic acid (47,64).

Formvar resin has been the polyvinyl acetal plastic most widely used for the preparation of support films (84). Formvar films are much stronger mechanically than those of Parlodion so that less drift occurs (14,47,84) and are less volatile (15 to 40% mass loss) under the electron beam (44), resulting in lower instrumental contamination rates (84). They conduct electrons much more readily so that surface electrostatic charge buildup is minimal, and less film disruption due to mutual charge repulsion occurs. Their disadvantages in comparison to Parlodion are greater. Formvar films are more difficult to prepare (14,47,84); common problems include surface roughness and a lack of uniformity in the film thickness, failure of films to detach from the casting surface, and the formation of small holes and patterns in the prepared supports (47,84). Due to the hydrophobicity of the plastic, aqueous negative stain preparations do not spread well over acetal supports, and proteins do not adsorb as well (47,61,84). Formvar films tend to char, rather than sublime, under the electron beam, and the resulting carbonization detracts from the specimen fine detail and contrast (94). Patchy backgrounds with or without circular surface patterns often result during negative staining, as a result of poor wetting of the film surface or uneven film drying, respectively (47,84).

Prepared solutions of Formvar in ethylene dichloride, chloroform, or dioxane break down quickly into free hydrochloric acid, even when protected in a dry, dark, cool storage area (47,64). Dawes (25) has suggested the use of Formvar solutions less than forty-eight hours old.

Polyvinyl butyral resins (Butvars B-76 and B-98) demonstrate most of the desirable properties of Parlodion and Formvar polymers. Their greater content of hydroxyl residues than Formvar imbues Butvars (especially B-98) with a higher degree of hydrophilicity and thus spreading ability which is comparable to that of Parlodion supports. A mechanical strength which exceeds that of Formvar greatly reduces the likelihood of specimen drift and film rupture; the inherent adhesiveness of Butvar films insures good bonding between the films and the mesh of the grids, contributing to this effect. The smoothness and uniformity of the film background are similar to those of Parlodion, as is the ease of film manufacture. Although Butvar films tend to char under the electron beam like those of Formvar resins, instrumental contamination rates are likewise low (84).

Plastic films prepared from organic polymers are prone to decomposition, drift, and shrinkage when bombarded by the electron beam, with the resultant distortion of fine structure, reduced contrast, and obviation of high resolution photography as a result of specimen drift (44,53). Carbon films can be used to stabilize the plastic substrates upon which negatively stained specimens rest: pure carbon sublimes only at very high temperatures (greater than 3500 C) without preceding liquefaction, and is thus thermostable (47,53,61,84). Irradiation of carbon films in the electron microscope causes the activation and cross-linking of carbon layers with the specimen upon one side and the plastic support film upon the

other (63); the carbon layer can transfer heat and electricity away from the plastic support and reduce charging so that the latter is physically stabilized. An associated drawback is the fact that the combined film of plastic and carbon is thicker than a film composed purely of either, and transparency is reduced (47). The direct *in vacuo* evaporation of a thin monolayer (1 to 5 nm, electron absorbance of less than 2%) of carbon from heated point contact carbon rods onto the plastic surface of a coated grid accomplishes this purpose.

Carbon films may also be used to provide extremely thin monolayer (2 nm or less) grid supports to be used for examining specimens at very high magnifications (100,000X or greater) during high resolution work (14,47,61). Such films demonstrate excellent uniformity and stability (14,84). In addition, carbon films can be made very thin, to give supports which have a fine-structured, uniformly transparent, low contrast background as a result of their low atomic weight atoms. This virtually eliminates resolution problems caused by the elastic and inelastic scattering of electrons (14,47,53,81). Brittleness and a tendency to rupture while handling cause carbon films to be difficult and laborious to prepare, while their extremely hydrophobic nature precludes good spreading of particles in aqueous suspension and causes the generation of wetting artefacts, such as small blotches (47,61,84). Firm bonding of carbon films to the grid mesh can be difficult to accomplish (47,84). A greater rate of specimen contamination appears to occur when carbon surfaces are used. The background grain generated by film inhomogeneities is relatively stronger than that seen when plastic films are employed, and carbon surfaces are rougher in texture (61).

Hahn and Baumeister (44) have pointed out the advantages of using polysynthetic plastic films for negative staining. These include: 1) simplicity of production; 2) lack of need for additional stabilization by evaporated carbon layers; 3) lack of a requirement for the addition of undesirable wetting agents to the film surface, as hydrophilization can be accomplished by short doses of UV light; 4) increased stabilization as a result of beam irradiation, due to increased cross-linking of the film material with time, instead of degradation; 5) small mass loss and shrinkage (less than 5%), even with large electron doses, facilitating the observation of large unobstructed specimen areas; 6) high electron transparency due to low density, i.e., low mass thickness relative to geometrical thickness; and 7) low spatial variations of mass thickness and a smooth surface, providing for homogeneous negative stain distributions.

Plastic Support Film Production

Well-controlled support film fabrication procedures are necessary for the dependable production of supports with desirable properties. Freshly-prepared specimen supports that perform well in turn insure the consistent production of high quality electron micrographs (84).

Parlodion films are prepared from stock or working solutions made by dissolving weighed cut pieces of plastic in a suitable solvent (ethyl, amyl, or butyl acetate, acetone) to achieve the desired concentration (0.2 to 2%, w/v). The solutions are placed in a clean, dry, blackened or foil-wrapped stoppered glass container stored in a cool, dry place for 1 to 2 days, with occasional agitation. The dissolved solution is filtered through Whatman #1 filter paper and stored as before. Best results are obtained by using freshly-

prepared (24 to 72-hour-old) solutions, although the preparation can be stored as long as 4 weeks before replacement. A weighed amount of Formvar resin is similarly dissolved in a suitable solvent (ethylene dichloride, dioxane, or chloroform) to give the desired concentration (0.15 to 2%, w/v), stored and filtered as for Parlodion, and used from 4 hours to 1 week after preparation. The highest quality Formvar films are cast from solutions less than 2 days old. Butvar film solutions are made by dissolving powdered resin in ethylene dichloride or chloroform in concentrations ranging from 0.15 to 2%, w/v. Solutions are stored in glass bottles with aluminum foil-wrapped caps, as Butvar resins are adhesives and will cement caps into place. Plastic containers are unsuitable, as they may contaminate the solutions with their plasticizers (84). For all types of film solutions, storage glassware should be chromate-cleaned, well brushed and rinsed with distilled water to remove residues, detergent, dust, etc., and thoroughly dried in a hot oven. Solutions should be protected from moisture by Parafilming the caps, from extremes of temperature, and exposure to light (25,47,84).

Plastic support films are made by casting the film solution onto a distilled water, solvent-saturated distilled water, or glass surface, the latter two methods resulting in the strongest and most uniform films, with glass-formed supports being superior (14,47,53,61,84). All three types of plastic solution may be cast onto a distilled water surface from a 2 to 3% solution, although Formvar films are difficult to prepare this way (14,40,53). A typical procedure for this method is as follows. A 10 to 20 cm diameter circular glass dish, 3 by 4 inch staining dish, or detachable flat bed funnel is filled with filtered distilled water. The water is prepared by filtering singly

or doubly-distilled water in bulk (10 to 15 liters) through a Millipore filtration unit consisting of a stainless steel positive pressure vessel (3 gallon capacity) and a 142 mm sterilizing filter holder plus filter (0.22 or 0.45 μ), and storing the filtrate in 1 liter chromate-cleaned stoppered glass flasks (61). After filling the flotation vessel with water to its edge, a large (100 mesh per inch) circular or rectangular screen, glass slide, or piece of cut filter paper is placed on the container bottom, and the desired number of grids arranged upon this. One or two drops of plastic solution are allowed to fall onto the center of the water surface from a clean pipette, the drops allowed to spread out by surface tension, and the solvent left to evaporate leaving a thin solid plastic film floating upon the water. Wrinkles are prevented from forming in the film by covering the flotation vessel with a Petri dish cover as the solvent volatilizes. The first film cast is stripped off with a needle or forceps and is used to clean the water surface. A second film is prepared in the same way and mounted onto the grids; the support (mesh or slide) with the grids upon it is lifted up through the floating film or the flotation water is siphoned or drained off through a basal tap to lower the film onto the grids. Grids can also be placed directly upon the floating film and the preparation picked up as described below. The coated grids are partially dried by wicking off the excess water with filter paper and storing them in a dry, clean, dust-free place (e.g., in a clean Petri dish placed in a pumped-out desiccator) (14,40,61).

Plastic support films may be cast onto standard 1 by 3 inch glass microscope slides or freshly-split mica (14,47,53,61,84). Freshly-cleaved mica affords the best surface substrate because of the cleanliness, homogeneity, and atomic smoothness of its exposed

surface (53,84). Precleaned slides free of dirt, dust, and glass chips are usable, although they can carry surface residues and impurities (surfactants, oils) from the cleaning process, and therefore may not be uniformly wettable; they may also produce films that contain artefacts or that are difficult to recover (84). Residues may be removed by soaking the slides for 24 hours in aqua regia, rinsing with filtered deionized water, draining the excess moisture off onto filter paper, and air-drying and storing them in a dust-free environment prior to use. Clean glass slides are uniformly wettable and residual water drains without beading, especially on the slide edges. Siliconized lens tissue can also be used to clean glass slides. Chromate cleaning is not acceptable because slides cleaned too well result in difficulty during film stripping (61,84). Cleaned slides may be lightly coated with detergent to aid film stripping, although many detergents solubilize in the film solutions and will cause the formation of weak, brittle films. The edges and one-half of one side of a previously cleaned slide can be lightly waxed with a suitable detergent (e.g., Teepol, Brij-58) or surfactant (e.g., sodium dialkylsophosuccinate, glycerol), and the excess lightly polished off with a lint-free twill cloth, chamois leather, or velvet, to leave behind a thin, fine, even surface film of detergent (14,84). Precleaned or cleaned slides with or without detergent coating are dipped into the plastic film solution for 10 to 15 seconds, the excess immediately and thoroughly drained from the slide end onto filter paper, and the plastic-coated slide allowed to dry standing or suspended in as near to vertical position as possible in a protected place. Drying should occur for one-half to one hour before flotation (47,84). Alternatively, the film solution is rapidly pipetted onto a horizontally-held slide from

one end to nearly the other, allowed to adsorb for several seconds, drained, and dried. Prior to draining and drying, coated slides may be held or hung in a slide holder in the vapor-saturated space above the film solution for one-half to one minute, and the excess drained by touching the slide corner to the vessel wall. This step results in thinner, more uniform films, as the film coat is kept running and the final drying retarded by the solvent vapor; more of the film solution coating the slide is removed upon draining (84,94).

After drying upon the slide, the plastic film is severed along the edges of the coated slide surface by scraping the edges with a sharp knife, scalpel, razor blade, or spatula edge, or scored near the edges and across the slide with the tips of a pair of forceps. The film can also be scored into small squares and these floated off as individual pieces; this is advantageous because film areas can be selected and film damage during grid removal is avoided. In all cases, the film is stripped from the slide by flotation onto a water surface. A clean 10 to 20 cm diameter glass circular dish or staining trough is filled with filtered distilled water to a point slightly above the level of the sides and the water surface freed of floating particles by sweeping it with a clean pipette, glass rod, or wet piece of lens tissue. The plastic film is stripped and floated by first breathing upon the film surface to initiate separation by water condensation, and presenting the coated slide at a low angle of incidence (15 to 20 degrees) to the water surface. The film-covered end of the slide is lowered to and then slightly below the water level so that surface tension forces lift the outer film edges and water is drawn into the film-glass interface to free the film and leave it floating on the water (14,25,47,84).

Grids or grid mounted nets are placed upon the floating film one-third to one-half (1 to 3 cm) of the way from the primary or thickest end. Grids are not placed near the film edges or on obviously heterogeneous areas of the film, and they should be mounted approximately 3 mm apart, matt side (punched grids) or either side (Athene-type grids) in contact with the film (47,84). The grid bars may be coated prior to actual mounting with film in order to aid stickiness by placing them upon bibulous paper and dropping 0.15% Butvar, 0.2% Formvar, or 1% polybutene 6000 in xylene onto the grids. The excess plastic solution rapidly moves into the paper, leaving a thin tacky coating only on the grid bars (40,61,84). Grids may also be etched for 2 minutes in concentrated HCl to improve their adhesiveness (61).

As a final step, the film-coated grids are retrieved from the water surface with a glass slide, wire or plastic net, Parafilm, or filter paper, by adsorbing the coated grids plus film mechanically onto the surface of the pickup device. The device is brought directly down onto the grids, pushed into the water, turned and retreated. Mechanical force and surface tension press the grids and their film onto the solid surface. Excess water is drained from around the coated grids by means of filter paper, and the coated grids allowed to dry in a clean, moisture-free environment (47,84).

When viewed from an oblique angle with incident light reflected from their surface, the best support films appear virtually transparent (200 Å) to dark gray (less than 500 Å) color. Films exhibiting a light bluish-silver (600 Å) to yellow (1000 Å) color are probably too thick. Film thickness can be varied by adjusting the concentration of plastic dissolved in the solvent, and solutions of higher plastic concentration give thicker films (47,84). Film strength can be roughly

assessed by gently pressing down upon the floating film with a pair of fine forceps; weak films will easily puncture or shatter, leaving holes or cracks, respectively, in the film surface. Good films will exhibit a point of impression with small wrinkles radiating from it which eventually disappear. Thick films are virtually impervious to such treatment (84). Weak, unstable films may result from a number of causes, including (67,84): 1) extreme thinness (low concentration of plastic in the film solution, grid openings are too large, etc.); 2) old plastic, solvent, or film solution (age, exposure to light, humidity, temperature extremes, etc.); 3) contamination of the plastic solution (oils, water vapor, dirt, dust, detergents, etc.); and 4) poor grid-film contact (uneven or warped mesh, film applied to a convex surface).

Carbon Support Film Production

Grid-mounted carbon support films are routinely and dependably prepared by the deposition of graphitic carbon *in vacuo* upon plastic, mica, glass, or detergent-coated glass substrates (84).

Briefly, carbon monolayers are evaporated by the following method (14,47,61,84). The apparatus commonly used for evaporation consists of a glass bell jar evacuated by a diffusion pump backed by a mechanical pump. Inside the bell jar are two hard graphitic carbon rods held in a fixed horizontal position by a pair of insulated posts. The posts are threaded by electrode wiring which connects the base of each rod with the power source. Both carbon rods are finely pointed (0.25 mm diameter), or more commonly one is pointed and the other is flattened (1.5 mm diameter); they contact each other and are held in place by a positive-pressure feed spring which applies force to one of the

pointed rods. Carbon vapor is generated in a high vacuum ($0.1 \mu \text{ Hg}$) by the resistive heating of the two graphitic carbon rods at their point of contact to produce an arc; power to the electrodes raises the temperature of the carbon rods to a point of incandescence where the sublimation temperature of carbon is exceeded at the point of contact of the rod faces and the smaller pointed rod with a lower thermal capacity becomes hotter and is burned away. The evaporated carbon is radiated as small particles in all directions from the point of contact of the two opposing rods, and the better the vacuum attained, the finer the resulting carbon spray. The thickness of the deposited carbon film is crudely measured by placing a clean piece of white glazed porcelain with a drop of fresh vacuum pump oil upon it near the specimen. A film approximately 5 nm thick has been evaporated when the uncovered porcelain just detectably turns a faint brown, and a film nearly 10 nm thick has been formed when the uncovered porcelain turns a light chocolate brown compared to the white oil-covered porcelain. A current of approximately 30 amp at 15 volts is passed through the electrode cables for 0.5 to 2 seconds to produce these results when the specimen lies 10 to 15 cm from the point source. An alternating current power source used to produce the arc providing the carbon vapor gives carbon films possessing lower wettability. Direct current sources will give films with a hydrophilicity comparable to that of Butvar; unfortunately these films tend to expand and sag across grid openings to give rippled, nonuniform surfaces. A solution to this problem is to deposit a direct current source carbon film of 1 to 15 nm thickness over an alternating current source carbon film of 1 to 15 nm thickness which results in a strong, rigid, hydrophilic carbon support film.

A simple, rapid technique for the production of general-purpose carbon films involves applying a coating (detergent or surfactant) to the surface of a clean glass slide or split mica sheet, polishing off the excess, vacuum-evaporating carbon (5 to 10 nm thick film) onto the detergent-coated side of the slide, and stripping and floating the film onto a water surface. The film may be scored into tiny squares prior to stripping or left intact. The floating carbon film is picked up by bringing the forceps-held grids up through the film or film squares, or by lowering the film onto grids by draining away the flotation liquid. The coated grids are dried on filter paper (14,40,47,46,61). Carbon evaporated onto glass surfaces is more difficult to recover by stripping. Glass films are also fragile and tend to shatter when stripped and/or are easily ruptured during the mounting operations. It is more difficult to firmly bind such films to grid surfaces and the inhomogeneities of the glass surface may be replicated (40). Freshly-cleaved mica produces films that are also fragile, but films are easier to strip from mica and are more uniform and anatomically smooth. Carbon films formed on both substrates are less wettable than those formed on plastic (61,84).

Carbon films prepared by the evaporation of carbon onto plastic surfaces are superior to plain glass or mica films, and they are more reliably produced (84). The advantages of using a plastic base include the production of extremely thin films, the obtainment of a more wettable carbon surface, and the firm bonding between plastic base films and grid mesh. Plastic films offer the disadvantages of less smooth and uniform carbon films due to the replication of the plastic surface structure; fabrication procedures are also more tedious. Parlodion and other nitrocellulose plastics, Butvar, and Formvar may be used as

surface substrates, but Formvar is superior. Parlodion-based films are less desirable because the rupture of the evaporated carbon film occurs over a substantial number of grid openings during solvent treatment; this event is theorized to be the result of the swelling of the plastic substrate prior to solubilization (84).

Plastic support films are prepared and mounted upon grids in a conventional manner, allowed to dry thoroughly, placed within a vacuum evaporator, and a 2 to 5 nm thick carbon coat deposited. The doubly-coated grids are transferred individually carbon side up to an extracting device to remove the underlying plastic substrate. The extractor is an apparatus for gently washing the grid preparations with the solvent used to dissolve the plastic resin. It consists of a 150 ml beaker with a wick made from a roll of bibulous paper stood upright in the beaker center within a glass cylindrical stand. The wick should be long enough to extend slightly above the plane of the beaker top. The beaker is closed by means of a circle of filter paper which contacts the top of the wick and is large enough to extend well beyond the rim of the beaker. The coated grids are placed upon the filter paper cover and enclosed themselves by a clean Petri dish lid. Solvent placed in the bottom of the beaker moves by capillary action up the wick and along the filter paper cover. Plastic film removed from the grids by the solvent is deposited towards the periphery of the filter paper where the solvent evaporates. The extent of washing is controlled by the amount of solvent (50 to 75 ml) placed in the bottom of the beaker (84). Alternatively, the carbon-coated plastic filmed grids can be washed by placing them upon a stainless steel mesh or wire gauze circle, and carefully lowering them into a flat-bed funnel containing plastic solvent for a time period ranging from a few

minutes to several hours (14,47,61). Depending upon the duration of exposure of the grids to the solvent, all of the plastic film can be dissolved away, or just that plastic between the grid bars, leaving plastic residue between the grid bars and the carbon film, thus insuring a firm bond between carbon and grid (14,47,61,84).

Oliver (84) recommends benzylamine tartrate, an organic glass, as an excellent substrate for forming carbon films. In the procedure used, the grid surface is fully exposed to a continuous film of carbon layered over the copper and the benzylamine tartrate surface, thus providing a firm bond plus a smooth carbon surface. A small shallow sieve of 200 mesh woven stainless steel cloth 1 to 2 inches in diameter is heated and melted benzylamine tartrate is applied with a spatula. The mesh of the sieve is then exposed to radiant heat from a hot plate to spread the molten substrate into a thin uniform layer (0.5 mm thick) which embeds the sieve wires; heating continues until all bubbles are eliminated. The sieve is allowed to cool, grids are placed within the sieve mesh openings upon the substrate surface, the sieve held 1 to 2 mm above a warm hot plate, and the substrate remelted so that the grids float upon the molten surface. The molten benzylamine tartrate penetrates the grid openings so as to be flush with the grid bar surfaces without wetting them, and the grids are inlaid with their metal surface left exposed. The preparation is cooled, carbon is evaporated upon the grids and the substrate surface, and the apparatus is extracted for several hours with methanol solvent to remove benzylamine tartrate.

Applications of Negative Contrast Electron Microscopy (NCEM)

General Biological Applications

Negative staining sufficiently improves contrast in biological material to permit the structural details of small particles, macromolecular complexes, and individual macromolecules to be visualized directly. As such, this method is capable of providing information about fine details of structure comparable to or better than that given by ultrathin sections, surface replicas, positive staining, or heavy metal shadowing (39). The original method of negative staining was developed for primary application to the study of bacterial, animal, and plant viruses and their components, but has found widespread use and firm establishment in areas outside of its main field of concentration, virology (39,84).

Small particulate or macromolecular biological specimens distinct from viruses to which NCEM has been applied include bacteria, protozoa, mycoplasma, chlamydiae, spermatozoa, muscle fibers, collagen and elastin fibers, fibrin, cellulose, glycogen, flagellae, fimbriae, pili, cell walls and membranes, insect tracheoles, ciliary rootlets, and cilia. Protein molecules, including native enzymes and crystals, ordered aggregates, oligomeric assemblages, and multichain or multi-enzyme complexes (e.g., catalase, hemoglobin, the pyruvate dehydrogenase complex, etc.) and various lipids (e.g., saponin, lecithin, and cholesterol) have been viewed (39,51,52,61,84). Concomitant with the rapid development of techniques for the fractionation of subcellular organelles and particles from different cells and tissues, negative contrasting has been used in the study of such isolated submicroscopic components as mitochondria, ribosomes, polysomes, nucleoli, whole

chromosomes, microtubules, nuclear envelopes, nerve ending vesicles, myelin fragments, acrosomes, and nucleic acid strands (39,49,51,52, 53,61). Additionally, it has been employed in the examination of whole cell mounts (48,53), frozen thin cell sections (1,53), and crude cell fragments (2,53,86) and lysates (2,3,53,105), with success.

General Virological Applications

The major contribution of NCEM has been towards the study of suspensions containing purified or partially purified virus particles and their isolated components. The general subunit arrangement of many simple viruses possessing icosahedral, helical, and complex symmetry in their protein shells has been established, and the structure of the envelope and internal nucleocapsid portions of membrane-bound viruses has also been elucidated (46,50,51,52,53).

As the functional roles of morphologically distinct and important substructures of bacterial, animal, and plant viruses have been deduced and clarified, so have been other diverse viral products (e.g., enzymes) pertinent to the solution of genetic, biochemical, and biophysical problems. The endproducts of viral degradation and viral *in vitro* self-assembly systems (47), the stages of interaction of viruses with host cell exterior, cytoplasmic, and nuclear membranes, and with virus-specific antibody molecules have been systematically explored (51,52,53).

Finally, NCEM has been valuable in purification procedures for ascertaining the homogeneity and degree of stage purity, for appraising the efficiency of preparative techniques, and for locating small amounts of morphologically recognizable virus or viral structures within fractionation schemes which otherwise might escape observation

by other means (e.g., sedimentation or electrophoresis) (46,84).

Quantitative or semi-quantitative methods of virus particle counting have also been coupled with negative staining technique (13,52,53,55).

Applications of NCEM to Human Virology

As more rapid methods of diagnosing viral infections have been introduced (e.g., immunofluorescence technique), the value of the virology laboratory has also increased for the clinician in the prompt and accurate establishment of the etiologic cause of infectious disease. Concomitantly, NCEM has found a limited but effective role in the diagnosis of viral infections of human origin by means of the detection and morphological identification of viruses found in clinical specimens (27).

In the realm of human virology, the feasibility of using the electron microscope for the examination of submitted clinical virology specimens was first demonstrated in the late 1940's by Nagler and Rake (80) and Van Rooyen and Scott (111) in their investigations of the contents of vesicular and pustular material obtained from patients with generalized smallpox, contagious pustular dermatitis, chickenpox, and herpes zoster. In specific and nonspecific fluids and ground crusts purified by differential centrifugation, they were able to demonstrate and differentiate the characteristic virions of the pox-virus and herpesvirus groups by means of gold shadowcasting. They suggested that the electron microscope could be used in the future as a tool in the diagnosis of variola, vaccinia, and varicella infections. Soon after this, many other clinical specimens were shown to contain intact and recognizable virus particles under direct shadowcast examination. Evans and Melnick (30) photographed chromium-shadowed varicella

and herpes zoster virus in crude preparations made from clarified vesicular and cerebrospinal fluids, and noted their similarity to each other and to herpes simplex particles. Strauss et al. (103) first reported the crystalline arrangements of spherical virus-like bodies in extracts of ground intranuclear inclusions from skin papillomas seen after chromium shadowcasting.

The introduction in 1959 (15) of a simple method for routine negative contrast electron microscopy (NCEM) enabling the rapid and precise identification of virus particles proved to be a great asset as a clinical diagnostic method. Following Horne and Nagington's negative staining examination of poliovirus-infected HeLa cell lysates (48), Williams et al. (114,115) in the early 1960's were among the first to apply the method of Brenner and Horne (15) to pooled fluid extracts of vesicular lesions caused by herpes simplex, herpes zoster, varicella, and primary smallpox, plus those of the benign cellular hyperplasias of molluscum contagiosum and verrucae vulgaris, plana, and palmaris et palmaris (warts) with marked success. These researchers pointed out the ready and precise group categorization of viruses that the method offered, and that it would also be of value in the future clinical diagnosis of difficult or atypical viral skin lesions. Nagington (79) used NCEM in the diagnosis of several cases and outbreaks of cowpox, herpes simplex, varicella, and papovavirus infections using crusts, vesicular fluids, and tissues as specimens. He gave an encouraging report on the success and applicability of the technique, especially in certain instances, including infections caused by viruses that could not be isolated by cell culture technique or which grew slowly. Cruickshank (23) likewise rapidly pinpointed the etiologic agent in a survey of recent smallpox cases, and was easily able to

distinguish these infections from clinically similar syndromes caused by chickenpox, herpes zoster, or herpes simplex. He commented on the success of the simple rapid technique of NCEM, and suggested that concentration and other time-consuming methods of preparation seemed unnecessary. The use of egg inoculation (e.g., via the chorioallantoic membrane route), gel-diffusion, and direct microscopy of stained smears as supportive tests was also offered. Macrae et al. (70) and Long et al. (66) confirmed the usefulness of NCEM in the rapid differential diagnosis of poxvirus and herpesvirus exanthems. Long et al. stressed the suitability of lesion fluid and crusts for smallpox and crusts for herpesvirus NCEM group identification, and that virus chorioallantoic membrane isolation was also needed for specific identification of different members of the poxvirus group. NCEM appeared to be a superior method for herpesvirus recognition, due to the marked lability of the virus. Macrae et al. also advocated the use of gel-diffusion for more specific virus identification. Both authors agreed upon the validity of NCEM for speedily pinpointing outbreaks of imported contagious smallpox in which the source of infection was not clearly established, or the first cases missed.

Doane et al. (26) first extended the use of NCEM to the rapid electron microscopic diagnosis of paramyxovirus respiratory infections. Parainfluenza, suspected mumps encephalitis, measles, and Newcastle disease viruses were rapidly detected and identified in nasopharyngeal secretions and cerebrospinal fluid of cases of acute laryngotracheitis. They maintained that these types of clinical specimens usually contained sufficiently high titers (greater than 10^8 TCID₅₀ per ml) of morphologically recognizable particles to be detected by NCEM, and that this method was of even greater value for viruses isolated in

cell culture. They felt that NCEM and/or hemagglutination and hemagglutination inhibition tests afforded the most rapid protocol for diagnosis of paramyxovirus disease. Joncas et al. (59) applied NCEM to clinical specimens in a controlled study of lower respiratory tract infections (croup, bronchiolitis, and bronchopneumonia) confined to children. Similarly, they were able to detect parainfluenza, respiratory syncytial, and *Mycoplasma pneumoniae*-like organisms, in clinical specimens or from cell culture, although adenovirus and picornavirus (polio, Coxsackie) presence later demonstrated by complement fixation serology and/or direct virus isolation was not confirmable by NCEM. Doane et al. (27) later reported the successful use of NCEM in the examination of fluid specimens (cerebrospinal fluid, vesicular and pustular fluids, throat and eye washings, nasopharyngeal secretions), small pieces of biopsy or autopsy material, cell culture supernates, lysed cell culture pellets, and contaminated primary cell lines. They also thought that a variety of other clinical specimens were worth investigation by NCEM, including urine (measles, mumps, rubella, vaccinia, adeno-, cytomegalo-, and Coxsackie virus) and erythrocytes (measles, cytomegalovirus), plus specimens previously processed by ultrathin sectioning, such as brain tissue from cases of acute necrotizing encephalitis, progressive multifocal leukoencephalopathy, and subacute sclerosing panencephalitis. They were unable to find a high concentration of enteroviruses in nasopharyngeal secretions or stool suspensions, which constituted an important limitation, as these viruses comprised a large proportion of cell culture isolates. A combined approach of NCEM with cell culture isolation was seen as desirable and a routine method of approach to clinical specimens with NCEM discussed.

The NCEM examination of fecal extracts has received a great deal of interest and application. Bishop et al. (10) used NCEM of diarrheic stools extracted with fluorocarbon and differentially centrifuged to reveal particles resembling rotavirus in children suffering from acute nonbacterial gastroenteritis. They stressed the timing of collection in relation to the onset of illness as being critically important for the detection of virus by electron microscopy. Chrystie et al. (20) reported rotavirus particles in specimens obtained from a diarrheic outbreak occurring in a maternity ward and in a newborn special care unit. They suggested that adult maternal carriers excreting virus amounts below the detectable threshold for NCEM were responsible. Furthermore, the technique proved to be valuable not only in clinically overt cases, but also in those that were mild or asymptomatic. Flewitt et al. (33) also found reovirus in stool specimens obtained from cases of acute infectious gastroenteritis in children. Flewett et al. (34,35) and others (18,72) have described recognizable particles of parvo- or picornaviruses, adenoviruses, coronaviruses, and myxoviruses, plus virions resembling those of myxovirus, coronavirus, and as yet unclassified virus groups. Pennington and colleagues (89) have found NCEM useful in the direct preliminary examination of material taken from skin lesions, feces, and eye infections, all caused by adenovirus, herpesvirus, or vaccinia. Eye infections often involved viruses causing cytopathic effects in cell culture, and routine preliminary examination of infected cell cultures by NCEM enabled rapid diagnosis plus a considerable saving of time and expense.

Applications of NCEM to Veterinary Virology

The use of NCEM in the rapid diagnosis of clinical specimens obtained from animals exhibiting viral disorders has likewise proven successful in veterinary medicine (29,38,68,100).

In a short research note, Spradbrow (99) became one of the first investigators in veterinary medicine to report the use of simple NCEM techniques for the rapid identification of animal viruses of veterinary significance. He emphasized the fact that NCEM of virus particles rendered a direct, easy, fast visual observation of the diagnostic characteristics of the etiologic agent(s). Moreover, simpler, more pragmatic techniques of preparation than differential or density gradient centrifugation had evolved to demonstrate cell-associated virus, namely, frozen thin sections of cell pellets and distilled water lysis of virus-infected cell monolayers. In a subsequent study utilizing the simple preparatory methods of Almeida and Howatson (1) and Almeida et al. (3), Spradbrow and Francis (100) grew bovine enterovirus, reovirus type 3, infectious bovine rhinotracheitis virus, equine herpesvirus, bovine adenovirus, and parainfluenzavirus type 3 in various animal cell culture lines and examined the distilled water lysates of the trypsinized cell pellets by NCEM for the presence of virus. Characteristic virus particles were detectable in all lysates with or without further dilution and/or cold storage of the lysate material. They concluded that the use of NCEM in this way for viral diagnosis could play a significant role in veterinary medicine in the future.

Davis et al. (24) clinically applied NCEM to the examination of crusts and vesicle fluids collected from pseudocowpox infections of calves and humans to obtain a rapid provisional diagnosis. Together

with the results from clinical symptomatology and pathological examinations of biopsy material, they were able to distinguish these lesions from those caused by members of the "rectangular" poxvirus group (vaccinia, variola, cowpox, and molluscum contagiosum), but not within the paravaccinia virus group by NCEM alone. Cooper (22) diagnosed poxvirus infections of peregrine falcons by NCEM of ocular discharges and skin scrapings and concluded that the method was superior to live bird inoculation or chorioallantoic membrane isolation.

Gibbs and Johnson (38), in a study on the differential diagnosis of bovine virus mammillitis, found NCEM of emulsified scabs and scrapings or vesicular fluids taken from infected herds to be a rapid and reliable technique when compared with methods of cell culture isolation. They were able to detect and differentiate particles of papovavirus, herpesvirus, cowpoxvirus, and pseudocowpoxvirus agents responsible for causing acute infectious disease. After pointing out the attendant advantages and disadvantages of NCEM in viral diagnosis, they further suggested the applicability of the technique to foot and mouth disease, bovine papular stomatitis virus and viruses of more exotic diseases, including rinderpest, bluetongue, Neethling, Allerton, and Lumpy Skin disease.

Marsolais et al. (71) harvested allantoic fluid and chorioallantoic membranes from embryonated hens' eggs inoculated with organ tissue (trachea, lungs, liver, brain, spleen, and bursa) homogenates of birds infected with infectious bronchitis virus. Using the method of Doane et al. (27), they prepared negatively stained carbon-Formvar-coated grids and under direct electron microscopy found morphologically recognizable coronavirus in fluids from first or second passage levels,

but rarely in tissue extracts. In comparison and in parallel, virus was detectable by pathological changes occurring in inoculated chicken embryos only at the fourth or fifth passage levels.

McFerran et al. (68), in a thorough investigation of the uses and misuses of NCEM in veterinary viral diagnosis, examined a diverse series of specimens for the presence of virus, including skin lesions, vesicular fluid, warts, egg inoculates, and cell culture lysates. Direct NCEM examination of clinical and laboratory material rapidly yielded herpesvirus, paramyxovirus, adenovirus, reovirus, picornavirus, and parvovirus as causative agents of infection. Surprisingly, some specimens yielded mixtures of two different viruses when initially only one agent was suspected. Furthermore, small picornaviruses and papovaviruses were found hard to differentiate by NCEM alone, as were viruses within a major virus group. They suggested routine use of NCEM in the examination of allantoic fluid, cell culture isolates, and skin, upper respiratory, and post-mortem specimens.

A number of other studies have substantiated the use of NCEM in veterinary medicine. Scott et al. (96) used NCEM for the rapid examination of ground septa taken from cases of porcine inclusion-body rhinitis caused by herpesvirus, in contrast to conventional methods of paraffin and ultrathin sectioning of the nasal mucosa, and found it superior. England et al. (28) diagnosed herpesvirus and adenovirus infections of horses and cattle by direct NCEM of nasal swabs from antemortem respiratory infections. The pellet obtained from the ultracentrifugate of swabs placed in PSS Ringer's Lactate was negatively stained; adenovirus was found with a greater incidence of success than herpesvirus, due to its hardness and the nature of its cellular release. Van Kammen and Spradbrow (110) identified poxvirus particles in tracheal

cell scrapings, swabs, scabs, and exudates from laryngotracheitis cases, by NCEM and by gel-diffusion. England et al. (29) processed cases of neonatal calf diarrhea by NCEM for viruses and could find coronavirus, reovirus, picornavirus, and parvovirus, plus assorted phages. They also found that NCEM could morphologically demonstrate virus particles in rare cases where immunofluorescence and virus isolation could not, and therefore felt that it was more sensitive and reliable. Bass and Sharpee (9) were able to observe equine coronavirus particles in fecal specimens and intestinal contents obtained from cases of acute infectious gastroenteritis. Negatively stained reovirus found in the diarrhea of lambs suffering from gastroenteritis has been described by Snodgrass and his associates (98) and confirmed by immunofluorescence and immunoelectron microscopy. Similar particles were observed in porcine transmissible gastroenteritis by Rodger and Craven (95) and McNulty et al. (69) in calves' feces, in addition to coronavirus, myxovirus, and *Mycoplasma*-like structures, in the case of the latter.

The Practicality of NCEM as a Means of Viral Diagnosis

The usefulness of NCEM for the diagnosis of viral infections by examining clinical specimens and/or cell culture fluids and lysates has been acclaimed by many investigators. Universally, it is agreed that the technique offers the advantages of rapidity, sensitivity, and simplicity (23,24,27,28,29,38,59,66,68,70,79,96,100,110,115). Accurate and reproducible results are commonplace, while complex and lengthy procedures (e.g., differential centrifugation, thin sectioning) which can change virus morphology are avoided (23,29,38,59,71,100,115).

The precise fundamental morphological characteristics of the viral etiologic agent(s) can be determined by direct visual impression, e.g., size, shape, and symmetry; number, type, and arrangement of capsomers; presence or absence of an envelope (27,28,29,38,66,79,100,115). As McFerran et al. (68) and others (28,66) have aptly pointed out, conclusive results are obtained by the observation of one or more unequivocally identifiable (i.e., well-stained and well-resolved) particle(s). Provisional results, moreover, are possible from seeing incomplete, but typical, virus structures or variants (66,68).

Less obvious advantages have been expressed by other researchers. A differential diagnosis can be made from lesions or cytopathic effects that are typical of more than one virus group (27,38,66,68). NCEM can be applied to difficult, atypical, or bizarre viral lesions or mild, virtually asymptomatic cases (7,66,115). It has been useful in detecting especially labile viruses such as herpes virus (28,66,79), slow- or irregularly-growing viruses (7,24,27,38,79), or viruses that have been inactivated (66), that cannot be grown from clinical materials (24,79), that cannot be grown in currently available cell or tissue culture systems (7,27,79), or that demonstrate few or no observable cytopathic or histopathologic effects (27,68). Cell culture contaminants (e.g., SV 40, reovirus, paramyxovirus, enteroviruses, mycoplasma) are easily detected by NCEM so that primary/continuous cell and tissue culture lines can be monitored and kept free of such adventitious agents, either by examination before culturing or prior to the inoculation of mature monolayers with clinical specimens (5,27,59,68). A rapid presumptive diagnosis can be made in suspected new outbreaks of routine or exotic diseases from the moment the first cytopathic effects are visible in cell culture, or immediately after clinical specimens

are submitted (22,38,59,66,70,79,100). The rapidity of diagnosis in all instances allows easy confirmation or revision of the initial suspicions as to the type of etiologic agent(s) (27), and can suggest further testing (e.g., serology) (27,59,68), the institution of appropriate control measures such as immune globulin administration, the limitation of contacts, or complete isolation (7,27,29,70).

The shortcomings of NCEM diagnosis have also been analyzed. The lack of absolute specificity allows NCEM to distinguish between different virus particles belonging to different major virus groups, but generally not within a group of closely-related viruses (7,28,29,38,66,115). This may not matter therapeutically or for control measures (7,30,68), so that NCEM is probably most valid in cases where a virus morphological type is distinct (79). Relatively high virus titers (at least 10^6 particles per ml) are necessary in a specimen for observation by NCEM, and detectable virus counts are not always possible in submitted clinical materials (27,28,29,66,89). Neither are all viruses recognizable (e.g., leukemia viruses) or distinguishable from each other (e.g., enteroviruses) or cellular debris in negatively stained unpurified virus preparations (30,34,68). The clinical specimens examined must be fresh, fluid in nature (e.g., feces, vesicular fluid, nasal secretions, tissue extracts), from active lesions, and the lesion material must be such that the virus remains localized or concentrated, as in warts or scabs (28). The maturation processes of certain viruses may preclude their detection by NCEM: cell-associated virus, budding virus, structurally sensitive viruses (ruptured during preparation) all contribute smaller amounts of free, detectable virus than do viruses which cause cell lysis and a burst of extracellular virus (28,68). Negative NCEM findings do not necessarily imply a lack

of virus present (89), but could reflect a low titer specimen (27,28), the quantity of specimen or the type of specimen submitted, or the skill and the experience of the microscopist (23). Finally, the equipment required is complex, expensive, of high performance, necessitates the employment of trained personnel, and is not yet commonly available to all laboratories (7,38,68,79).

The importance of including relevant clinical, epizootiological, and pathological case data with the NCEM specimen to be examined has been stressed (24). Moreover, a combined approach of NCEM with other diagnostic tools (e.g., fluorescent antibody staining, cell culture isolation, serology) should provide confirmatory data.

LITERATURE CITED

LITERATURE CITED

1. Almeida, J. D., and A. F. Howatson. 1963. A negative staining method for cell-associated virus. *J. Cell Biol.* 16:616-620.
2. Almeida, J. D., and D. A. J. Tyrell. 1967. The morphology of three previously uncharacterized human respiratory viruses that grow in organ culture. *J. Gen. Virol.* 1:175-178.
3. Almeida, J. D., A. P. Waterson, and W. Plowright. 1967. The morphological characteristics of African swine fever virus and its resemblance to tipula iridescent virus. *Arch. Gesamte Virusforsch.* 20:392-396.
4. Anderson, N., and F. W. Doane. 1972. Agar diffusion method for negative staining of microbial suspensions in salt solutions. *Appl. Microbiol.* 24:495-496.
5. Anderson, N., and F. W. Doane. 1972. Microscopic detection of adventitious viruses in cell cultures. *Can. J. Microbiol.* 18:299-304.
6. Anderson, T. F. 1951. Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. *Trans. N.Y. Acad. Sci.* 13:130-134.
7. Banatvala, J. E., I. L. Chrystie, and A. J. E. Flower. 1975. Rapid diagnosis of virus infections. *Lancet* 2:79-80.
8. Barnard, J. E., and F. V. Welch. 1936. Fluorescence microscopy with high powers. *J. R. Microsc. Soc.* 56:361-364.
9. Bass, E. P., and R. L. Sharpee. 1975. Coronavirus and gastroenteritis in foals. *Lancet* 2:822.
10. Bishop, R. F., G. P. Davidson, I. H. Holmes, and B. J. Ruck. 1974. Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis. *Lancet* 2:149-151.
11. Black, L. M., C. Morgan, and R. W. G. Wyckoff. 1950. Visualization of tobacco mosaic virus within infected cells. *Proc. Soc. Exp. Biol. Med.* 73:119-122.
12. Borrel, A. 1904. Sur les inclusions de l'epithelioma contagieux des oiseaux. *C. R. Soc. Biol.* 57:642-650.

13. Bradley, D. E. 1962. A study of the negative staining process. *J. Gen. Microbiol.* 29:503-516.
14. Bradley, D. E. 1965. The preparation of specimen support films, pp. 58-74. *In* D. Kay (ed.), *Techniques for Electron Microscopy*, Blackwell Scientific Publications, Oxford.
15. Brenner, S., and R. W. Horne. 1959. A negative staining method for high resolution electron microscopy of viruses. *Biochim. Biophys. Acta* 34:103-110.
16. Brenner, S., G. Streisinger, R. W. Horne, S. P. Champe, L. Barnett, S. Benzer, and M. W. Rees. 1959. Structural components of bacteriophage. *J. Mol. Biol.* 1:281-292.
17. Buist, J. B. 1887. *Vaccinia and Variola: A Study of Their Life History*. J. and A. Churchill, Ltd., London.
18. Caul, E. O., W. K. Paver, and S. K. R. Clarke. 1975. Coronavirus particles in faeces from patients with gastroenteritis. *Lancet* 1:1192.
19. Caul, E. O., C. R. Ashley, and S. I. Egglestone. 1977. Recognition of human enteric coronaviruses by electron microscopy. *Med. Lab. Sci.* 34:259-263.
20. Chrystie, I. L., B. Totterdell, M. J. Baker, J. W. Scopes, and J. E. Banatvala. 1975. Rotavirus infections in a maternity unit. *Lancet* 2:79.
21. Claude, A. 1948. Studies on cells: morphology, chemical constitution, and distribution of biochemical functions. *Harvey Lectures* 43:121-164.
22. Cooper, J. E. 1969. Two cases of pox in recently imported peregrine falcons (*Falco peregrinus*). *Vet. Rec.* 85:683-684.
23. Cruickshank, J. G., H. S. Bedson, and D. H. Watson. 1966. Electron microscopy in the rapid diagnosis of smallpox. *Lancet* 2:527-530.
24. Davis, C. M., G. Musil, and J. A. Trochet. 1970. Electron microscopy for the rapid diagnosis of pseudocowpox and milker's nodule. *Am. J. Vet. Res.* 31:1497-1503.
25. Dawes, C. J. 1971. *Biological Techniques in Electron Microscopy*. Barnes and Noble, Inc., New York.
26. Doane, F. W., N. Anderson, K. Chatiyanda, R. M. Bannatyne, D. M. McLean, and A. J. Rhodes. 1967. Rapid laboratory diagnosis of paramyxovirus infections by electron microscopy. *Lancet* 2:751-753.

27. Doane, F. W., N. Anderson, A. Zbitnew, and A. J. Rhodes. 1969. Application of electron microscopy to the diagnosis of virus infections. *Can. Med. Assoc. J.* 100:1043-1049.
28. England, J. J., R. M. Barratt, E. A. Enright, and C. S. Frye. 1976. Diagnosis of viral respiratory infections by direct electron microscopic examination of nasal swabs, pp. 377-384. In *Proceedings of the 19th Annual Meeting: The American Association of Veterinary Laboratory Diagnosticians*. American Association of Veterinary Laboratory Diagnosticians, 6101 Mineral Point Road, Madison, Wisconsin.
29. England, J. J., C. S. Frye, and E. A. Enright. 1976. Negative contrast electron microscopic diagnosis of viruses of neonatal calf diarrhea. *Cornell Vet.* 66:172-182.
30. Evans, A. S., and J. L. Melnick. 1949. Electron microscope studies of the vesicle and spinal fluids from a case of herpes zoster. *Proc. Soc. Exp. Biol. Med.* 71:283-286.
31. Farrant, J. L. 1954. An electron microscopic study of ferritin. *Biochim. Biophys. Acta* 13:569-576.
32. Fernandez-Moran, H. 1956. Applications of a diamond knife for ultrathin sectioning to the study of the fine structure of biological tissues and metals. *J. Biophys. Biochem. Cytol.* 2 (Suppl.):29-30.
33. Flewett, T. H., A. S. Bryden, and H. Davies. 1973. Virus particles in gastroenteritis. *Lancet* 2:1497.
34. Flewett, T. H., A. S. Bryden, and H. Davies. 1974. Diagnostic electron microscopy of faeces. I. The viral flora of the faeces as seen by electron microscopy. *J. Clin. Pathol.* 27:603-608.
35. Flewett, T. H., H. Davies, A. S. Bryden, and M. J. Robertson. 1974. Diagnostic electron microscopy of faeces. II. Acute gastroenteritis associated with reovirus-like particles. *J. Clin. Pathol.* 27:608-614.
36. Fullam, E. F., and A. E. Gessler. 1946. A high speed microtome for the electron microscope. *Rev. Sci. Instrum.* 17:23-35.
37. Gaylord, W. H., Jr., and J. L. Melnick. 1953. Intracellular forms of pox viruses as shown by the electron microscope (vaccinia, ectromelia, molluscum contagiosum). *J. Exp. Med.* 98:157-171.
38. Gibbs, E. P. J., and R. H. Johnson. 1970. Differential diagnosis of virus infections of the bovine teat skin by electron microscopy. *J. Comp. Pathol.* 80:455-463.

39. Glauert, A. M. 1965. Factors influencing the appearance of biologic specimens in negatively stained preparations. *Lab. Invest.* 14:331-341.
40. Goodhew, P. J. 1972. Specimen preparation in materials science, pp. 7-180. *In* A. M. Glauert (ed.), *Practical Methods in Electron Microscopy*, Volume 1. North Holland Publishing Co., Amsterdam.
41. Gordon, C. N. 1972. The use of octadecanol monolayers as wetting agents in the negative staining technique. *J. Ultrastruct. Res.* 39:173-185.
42. Green, R. H., T. F. Anderson, and J. E. Smadel. 1942. Morphological structure of the virus of vaccinia. *J. Exp. Med.* 75:651-655.
43. Gregory, D. W., and B. J. S. Pirie. 1973. Wetting agents for biological electron microscopy. I. General considerations and negative staining. *J. Microsc. (Oxford)* 99:251-265.
44. Hahn, M., and W. Baumeister. 1976. Specimen supports for high and ultrahigh resolution electron microscopy, pp. 283-285. *In* J. A. Venables (ed.), *Developments in Electron Microscopy and Analysis*. Academic Press, Inc., New York.
45. Hall, C. E. 1955. Electron densitometry of stained virus particles. *J. Biophys. Biochem. Cytol.* 1:1-12.
46. Haschemeyer, R. H., and R. J. Meyers. 1972. Negative staining, pp. 100-147. *In* M. A. Hayat (ed.), *Principles and Techniques of Electron Microscopy: Biological Applications*, Volume 2. Van Nostrand Reinhold Co., New York.
47. Hayat, M. A. 1970. *Principles and Techniques of Electron Microscopy: Biological Applications*, Volume 1. Van Nostrand Reinhold Co., New York.
48. Horne, R. W., and J. Nagington. 1959. Electron microscope studies of the development and structure of poliomyelitis virus. *J. Mol. Biol.* 1:333-338.
49. Horne, R. W., and V. P. Whittaker. 1962. The use of the negative staining method for the electron-microscopic study of subcellular particles from animal tissues. *Z. Zellforsch. Mikrosk. Anat.* 58:1-16.
50. Horne, R. W., and P. Wildy. 1963. Virus structure revealed by negative staining. *Adv. Virus Res.* 10:101-170.
51. Horne, R. W. 1964. Some recent applications of negative-staining methods to the study of biological structure in the electron microscope. *J. R. Microsc. Soc.* 83:169-177.

52. Horne, R. W. 1965. Negative staining methods, pp. 328-355. In D. Kay (ed.), *Techniques for Electron Microscopy*. Blackwell Scientific Publications, Oxford.
53. Horne, R. W. 1967. Electron microscopy of isolated virus particles and their components, pp. 521-574. In K. Maramorosch and H. Koprowski (eds.), *Methods in Virology*, Volume 3. Academic Press, Inc., New York.
54. Horne, R. W. 1975. Recent advances in the application of negative staining to the study of virus particles examined in the electron microscope, pp. 227-274. In R. Barer and V. E. Cosslett (eds.), *Advances in Optical and Electron Microscopy*, Volume 6. Academic Press, Inc., New York.
55. Howatson, A. F. 1969. Electron microscopic procedures in virology, pp. 504-524. In K. Habel and N. P. Salzman (eds.), *Fundamental Techniques in Virology*. Academic Press, Inc., New York.
56. Huxley, H. E. 1956. Some observations on the structure of tobacco mosaic virus, pp. 260-261. In *Proceedings of the European Regional Conference on Electron Microscopy, Stockholm*. Academic Press, Inc., New York.
57. Huxley, H. E., and G. Zubay. 1960. Electron microscope observations on the structure of microsomal particles from *Escherichia coli*. *J. Mol. Biol.* 2:10-18.
58. Johnson, M. W., and R. W. Horne. 1970. Some observations on the relative dehydration rates of negative stains and biological objects. *J. Microsc. (Oxford)* 91:197-202.
59. Joncas, J. H., L. Berthiaume, R. Williams, P. Beaudry, and V. Pavilanis. 1969. Diagnosis of viral respiratory infections by electron microscopy. *Lancet* 1:956-959.
60. Kausche, G. A., E. Pfankuch, and H. Ruska. 1939. Die Sichtbarmachung von pflanzlichem Virus im Uebermikroskop. *Naturwissenschaften* 27:292-299.
61. Kay, D. 1976. Electron microscopy of small particles, macromolecular structures, and nucleic acids, pp. 177-215. In J. R. Norris (ed.), *Methods in Microbiology*, Volume 9. Academic Press, Inc., New York.
62. Knoll, M., and E. Ruska. 1932. Das Elektronenmikroskop. *Z. Phys.* 78:318-339.
63. Koelbel, H. K. 1974. Partially carbon coated support films - qualities and application. *Mikroskopie* 30:208-214.
64. Ladd, M. W. 1973. *The Electron Microscope Handbook: Specimen Preparation and Related Laboratory Procedures*. Ladd Research Industries, Inc., Burlington, Vermont.

65. Latta, H., and J. F. Hartmann. 1950. Use of a glass edge in thin sectioning for electron microscopy. *Proc. Soc. Exp. Biol. Med.* 74:436-439.
66. Long, G. W., J. Noble, Jr., F. A. Murphy, K. L. Herrmann, and B. Lourie. 1970. Experience with electron microscopy in the differential diagnosis of smallpox. *Appl. Microbiol.* 20:497-504.
67. McAlear, J. H. 1971. *Biological Electron Microscopy*. University of California, Berkeley.
68. McFerran, J. B., J. K. Clarke, and W. L. Curran. 1971. The application of negative contrast electron microscopy to routine veterinary virus diagnosis. *Res. Vet. Sci.* 12:253-257.
69. McNulty, M. S., W. L. Curran, and J. B. McFerran. 1975. Virus-like particles in calves' faeces. *Lancet* 2:78-79.
70. Macrae, A. D., A. M. Field, J. R. McDonald, E. V. Meurisse, and A. A. Porter. 1969. Laboratory differential diagnosis of vesicular skin rashes. *Lancet* 2:313-316.
71. Marsolais, G., L. Berthiaume, E. DiFranco, and P. Marois. 1971. Rapid diagnosis by electron microscopy of avian coronavirus infection. *Can. J. Comp. Med.* 35:285-288.
72. Mathan, M., V. I. Mathan, S. P. Swaminathan, S. Yesudoss, and S. J. Baker. 1975. Pleomorphic virus-like particles in human faeces. *Lancet* 1:1068-1069.
73. Mordoh, J., L. F. Leloir, and C. R. Krisman. 1965. *In vitro* synthesis of particulate glycogen. *Proc. Natl. Acad. Sci. U.S.A.* 53:86-91.
74. Morgan, C., and R. W. G. Wyckoff. 1950. The electron microscopy of fowl pox virus within the chorioallantoic membrane. *J. Immunol.* 65:285-295.
75. Morgan, C., S. A. Ellison, H. M. Rose, and D. H. Moore. 1954. Structure and development of viruses as observed in the electron microscope. I. Herpes simplex virus. *J. Exp. Med.* 100:195-202.
76. Morgan, C., S. A. Ellison, H. M. Rose, and D. H. Moore. 1954. Structure and development of viruses as observed in the electron microscope. II. Vaccinia and fowl pox viruses. *J. Exp. Med.* 100:301-308.
77. Morgan, C., H. M. Rose, and D. H. Moore. 1956. Structure and development of viruses as observed in the electron microscope. III. Influenza virus. *J. Exp. Med.* 104:171-181.

78. Munn, E. A. 1968. On the structure of mitochondria and the value of ammonium molybdate as a negative stain for osmotically sensitive structures. *J. Ultrastruct. Res.* 25:362-380.
79. Nagington, J. 1964. Electron microscopy in differential diagnosis of poxvirus infections. *Br. Med. J.* 2:1499-1500.
80. Nagler, F. P. O., and G. Rake. 1948. The use of the electron microscope in diagnosis of variola, vaccinia, and varicella. *J. Bacteriol.* 55:45-51.
81. Nermut, M. V. 1972. Negative staining of viruses. *J. Microsc. (Oxford)* 96:351-362.
82. Newman, S. B., E. Borysko, and M. Swerdlow. 1949. Ultra-microtomy by a new method. *J. Res. Nat. Bur. Stand.* 43:183-199.
83. O'Brien, H. C., and G. M. McKinley. 1943. New microtome and sectioning method for electron microscopy. *Science* 98:455-456.
84. Oliver, R. M. 1973. Negative stain electron microscopy of protein macromolecules, pp. 616-672. *In* C. H. W. Hirs and S. N. Timasheff (eds.), *Methods in Enzymology: Enzyme Structure*, Volume 21D. Academic Press, Inc., New York.
85. Palade, G. E. 1952. A study of fixation for electron microscopy. *J. Exp. Med.* 95:285-297.
86. Parsons, D. F. 1963. Negative staining of thinly spread cells and associated virus. *J. Cell Biol.* 16:620-626.
87. Paschen, E. 1906. Was wissen wir ueber den Vakzineereger? *Muench. Med. Wochenschr.* 53:2391-2393.
88. Pease, D. C., and R. F. Baker. 1948. Sectioning techniques for electron microscopy using a conventional microtome. *Proc. Soc. Exp. Biol. Med.* 67:470-474.
89. Pennington, T. H., E. A. C. Follett, and M. C. Timbury. 1975. Rapid diagnosis of virus infections. *Lancet* 2:182.
90. Porter, K. R., A. Claude, and E. F. Fullam. 1945. A study of tissue culture cells by electron microscopy. *J. Exp. Med.* 81:233-244.
91. Porter, K. R., and H. P. Thompson. 1948. A particulate body associated with epithelial cells cultured from mammary carcinomas of mice of a milk-factor strain. *J. Exp. Med.* 88:15-23.
92. Porter, K. R., and J. Blum. 1953. A study in microtomy for electron microscopy. *Anat. Record* 117:685-706.

93. Reissig, M., and S. A. Orrell. 1970. A technique for the electron microscopy of protein-free particle suspensions by the negative staining method. *J. Ultrastruct. Res.* 32: 107-117.
94. Ritchie, A. E. Unpublished observations.
95. Rodger, S. M., and J. A. Craven. 1975. Demonstration of reovirus-like particles in intestinal contents of piglets with diarrhoea. *Aust. Vet. J.* 51:536.
96. Scott, A. C., P. H. Lamont, M. S. Chapman, and J. D. J. Harding. 1973. Electron microscopy in the rapid diagnosis of inclusion-body rhinitis of pigs. *Vet. Rec.* 93:127-128.
97. Sharp, D. G., A. R. Taylor, D. Beard, and J. W. Beard. 1942. Study of the papilloma virus protein with the electron microscope. *Proc. Soc. Exp. Biol. Med.* 50:205-207.
98. Snodgrass, D. R., W. Smith, E. W. Gray, and J. A. Herring. 1976. A rotavirus in lambs with diarrhoea. *Res. Vet. Sci.* 20:113-114.
99. Spradbrow, P. B. 1968. Electron microscopy as an aid to the rapid identification of animal viruses. *Aust. Vet. J.* 44:427.
100. Spradbrow, P. B., and J. Francis. 1969. Electron microscopy as an aid to the rapid identification of animal viruses. *Vet. Rec.* 84:244-246.
101. Stanley, W. M., and T. F. Anderson. 1941. A study of purified viruses with the electron microscope. *J. Biol. Chem.* 139: 325-338.
102. Strangeways, T. S. P., and R. G. Canti. 1927. The living cell *in vitro* as shown by dark-ground illumination and the changes induced in such cells by fixing reagents. *Q. J. Microsc. Sci.* 71:1-15.
103. Strauss, M. J., E. W. Shaw, H. Bunting, and J. L. Melnick. 1949. "Crystalline" virus-like particles from skin papillomas characterized by intranuclear inclusion bodies. *Proc. Soc. Exp. Biol. Med.* 72:46-50.
104. Taylor, A. R., D. G. Sharp, D. Beard, J. W. Beard, J. H. Dingle, and A. E. Feller. 1943. Isolation and characterization of influenza A virus (PRP strain). *J. Immunol.* 47:261-282.
105. Tyrell, D. A. J., and J. D. Almeida. 1967. Direct electron-microscopy of organ cultures for the detection and characterization of viruses. *Arch. Gesamte Virusforsch.* 22:417-425.
106. Valentine, R. C. 1958. Quantitative electron staining of virus particles. *J. R. Microsc. Soc.* 78:26-29.

107. Valentine, R. C. 1959. The shape of protein molecules suggested by electron microscopy. *Nature (London)* 184:1838-1841.
108. Valentine, R. C. 1961. Contrast enhancement in the electron microscopy of viruses. *Adv. Virus Res.* 8:287-318.
109. Valentine, R. C., and R. W. Horne. 1962. An assessment of negative staining techniques for revealing ultrastructure, pp. 263-277. *In* R. J. C. Harris (ed.), *The Interpretation of Ultrastructure*. Academic Press, Inc., New York.
110. Van Kammen, A., and P. B. Spradbrow. 1976. Rapid diagnosis of some avian virus diseases. *Avian Dis.* 20:748-751.
111. Van Rooyen, C. E., and G. D. Scott. 1948. Smallpox diagnosis with special reference to electron microscopy. *Can. J. Public Health* 39:467-477.
112. Von Ardenne, M. 1939. The wedge sectioning method for producing microtome cuts of less than 10^{-3} mm thickness for purposes of electron microscopy. *Z. Wiss. Mikrosk.* 56:8-12.
113. Webb, M. J. W. 1973. A method for the rapid removal of sugars and salts from virus preparations on electron microscope grids. *J. Microsc. (Oxford)* 98:109-111.
114. Williams, M. G., A. F. Howatson, and J. D. Almeida. 1961. Morphological characterization of the virus of the human common wart (*Verruca vulgaris*). *Nature (London)* 189:895-897.
115. Williams, M. G., J. D. Almeida, and A. F. Howatson. 1962. Electron microscope studies on viral skin lesions. *Arch. Dermatol.* 86:290-297.
116. Williams, R. C., and R. W. G. Wyckoff. 1945. Electron shadow-micrography of virus particles. *Proc. Soc. Exp. Biol. Med.* 58:265-270.
117. Williams, R. C. 1953. A method of freeze-drying for electron microscopy. *Exp. Cell Res.* 4:188-201.
118. Williams, R. C., and H. W. Fisher. 1970. Electron microscopy of tobacco mosaic virus under conditions of minimal beam exposure. *J. Mol. Biol.* 52:121-123.
119. Woodruff, A. M., and E. W. Goodpasture. 1931. The susceptibility of the chorio-allantoic membrane of chick embryos to infection with the fowl-pox virus. *Am. J. Pathol.* 7:209-222.

ARTICLE

NEGATIVE CONTRAST ELECTRON MICROSCOPY
OF VETERINARY VIRUSES

By

M. R. Verrilli, A. W. Roberts, and G. R. Carter

SUMMARY

Representative viruses of each major morphological virus type were examined by negative contrast electron microscopy (NCEM) according to variables of negative stain type, concentration, and pH, plastic support substrate, and biological wetting agent. One per cent phosphotungstic acid (PTA), pH 6.5, plus Parlodion support films, with or without carbon strengthening, were found to provide suitable negative staining conditions for most viruses studied. Herpes virus particles were optimally contrasted with one per cent PTA, pH 7.0, and membrane-bound viruses could additionally be visualized by one-half per cent ammonium molybdate, pH 7.0, which appeared to give better membrane preservation than seen with PTA contrasting. One per cent PTA, pH 5.0, was used to negatively contrast certain nonenveloped RNA viruses, although one per cent PTA, pH 6.5, was equally good for others. Butvar support films compared favorably to Formvar supports, and both types of film were judged less preferable to Parlodion. Bacitracin afforded a superior spreading agent for negative staining purposes. Established parameters of NCEM were extrapolated to seven additional viruses commonly encountered in veterinary virology, and successfully applied to the examination of distilled water lysates of infected cell monolayers. Methanol precipitation was shown to be an effective concentration procedure for all major morphological virus types.

INTRODUCTION

In recent years, negative contrast electron microscopy (NCEM) has been applied towards the rapid diagnosis of many viral diseases encountered in veterinary medicine. A variety of clinical specimens,

including crusts (2), skin scrapings and vesicular fluids (11), allantoic fluids and chorioallantoic membranes (11,12), warts (11), nasal swabs (4) and ground nasal mucosa (15), upper respiratory secretions and tracheal scrapings (17), tissue homogenates (12), and diarrheic fecal material (3), have been successfully examined by this method, as well as cell culture fluids and distilled water lysates of virus-infected cell monolayers (4,11,16,17).

Although a variety of negative staining procedures and conditions has been employed for NCEM examination of clinical specimens and cell culture isolates, no routine methodology for negatively-contrasting commonly-encountered viruses has been established.

The purpose of this report is to describe the controlled observation of representative morphological virus types according to negative staining conditions of negative stain type, concentration, and pH, plastic support film, and biological spreading agent, and the application of these standardized conditions to other animal viruses. A simple, effective method for concentrating virus from dilute distilled water lysates is also described.

MATERIALS AND METHODS

Viruses - Viruses used in this study and the culture systems employed for their propagation are shown in Table 1. Control viruses represented the basic morphological and nucleic acid types, viz., non-enveloped DNA virus, enveloped DNA virus, nonenveloped RNA virus, enveloped helical RNA virus, and complex DNA virus. Cells used for virus multiplication were grown in Eagle's minimal essential medium with Earle's base supplemented with 0.5% lactalbumin hydrolysate,

sodium pyruvate, nonessential amino acids, and 10% bovine fetal serum (EMEM). Each milliliter of growth medium also contained 100 units of penicillin G, 100 µg of streptomycin sulfate, and 50 units of polymyxin B sulfate. Maintenance medium was the same, but contained only 2% bovine fetal serum. Distilled water lysates of virus-infected cell monolayers were prepared essentially by the method of Spradbrow and Francis (16). Briefly, inoculated cell cultures were examined daily for cytopathic effects, and when these were obvious (50 to 75% infection of the monolayer), the remaining cells were trypsinized from the glass surface. The cell suspension was centrifuged for 5 minutes at low speed (2000 x g) to pellet the cells, the pellet washed quickly and gently with distilled water to remove residual medium, and the cells resuspended thoroughly in 5 to 10 volumes of distilled water. After allowing cellular lysis to proceed at room temperature for 10 minutes, the lysate was rapidly frozen and thawed in an ethanol-dry ice bath several times and centrifuged again briefly at low speed to remove cellular debris. The clarified supernatant fluid was drawn off, distributed in 0.5 ml amounts into small screw-capped glass vials, and either examined immediately under the electron microscope or stored at -70 C. Allantoic fluid aspirated from infected embryonated chicken eggs was centrifuged briefly at low speed and treated as described for clarified cell culture lysates.

Methanol Concentration - Methanol precipitation of viruses was performed according to the method of Goodheart et al. (5). Chilled (2 C) aliquots of clarified distilled water lysates containing control virus types were divided in half, and one portion (0.25 ml) was diluted 100-fold to a 25 ml final volume with cold EMEM diluent prior to

methanol precipitation. The other portion was retained for direct electron microscopic examination. One-half volume of methanol cooled to at least -65 C was slowly and thoroughly mixed into the dilute infective fluids. The mixture was maintained at all times at a temperature of 2 to 3 C in an ice bath. Cold methanol was added slowly dropwise only as the mixture temperature remained stable. If the temperature exceeded 3 C, the further addition of methanol was halted until the temperature dropped below 3 C. After the methanol was completely added, the mixture was stirred for 3 hours at 2 C in an ice bath. The resulting precipitate was collected by centrifugation at 0 C in an IEC refrigerated centrifuge (Model B-20, fixed angle rotor) at 10,000 to 12,000 rpm for 20 minutes. The supernatant fluid was discarded and the cold pellets were quickly and gently resuspended in 0.25 ml of distilled water. The suspensions were allowed to warm to room temperature and to elute for 30 minutes. After centrifugation at 2 C in an Eppendorf Microcentrifuge (Model 3200/30, fixed angle rotor) at 12,000 rpm for 30 minutes to remove debris, the supernatant fluid was aliquoted into 2 equal volumes and placed into small screw-capped glass vials. These aliquots were either examined immediately under the electron microscope or stored at -70 C.

Negative Stains - The negative stains employed for virus contrasting included phosphotungstic acid (PTA, Matheson-Bell Scientific Co.), silicotungstic acid (STA, Polysciences), uranyl acetate (UA, Fisher Scientific Co.), cobaltous tungstate (CTA, gift of Dr. C. A. Baechler), and ammonium molybdate (AM, Mallinckrodt, Inc.). Weighed amounts of negative stain were dissolved in distilled water to give the desired final concentration (0.5, 1.0, 2.0, and 4.0%) and then titrated

(except UA) dropwise to the appropriate pH (pH 5.0, 6.5, 7.0, and 7.5) with 5N KOH. The pH-adjusted stains were filtered through 0.45 μ m Millex disposable syringe filter units (Millipore Co.) and stored in sterilized, chromate-cleaned, screw-capped glass bottles at room temperature. Bottle caps were wrapped tightly in Parafilm (Scientific Products) to guard against pH changes due to CO₂ dissolution, and the stain pH checked prior to use. Stains were replaced monthly.

Plastic Support Film - Materials used for manufacturing specimen support films to be used in negative staining included Parlodion (Ted Pella Co.), Formvar (Ted Pella Co.), and Butvar B-98 (Monsanto Chemical Co.) plastics. Film solutions were prepared by dissolving weighed amounts of cut Parlodion plastic strips or powdered Formvar or Butvar resin in the appropriate volume of solvent to achieve the desired concentration (0.25 or 0.5% Parlodion, 0.25% Formvar and Butvar) of polymer. Parlodion plastic was dissolved in amyl acetate (Fisher Scientific Co.) and Formvar and Butvar in ethylene dichloride (Fisher Scientific Co.). Film solutions were kept in chromate-cleaned screw-capped glass bottles wrapped in aluminum foil for 48 hours (Parlodion) or 4 to 6 hours (Formvar and Butvar) duration, in order to allow complete dissolution, and then filtered through Whatman #1 filter paper and stored as before. Fresh Parlodion and Butvar solutions were made weekly, and Formvar solutions were reconstituted every 2 days. Films were cast upon precleaned standard glass microscope slides essentially according to the method of Hayat (9), and floated off onto filtered distilled water. Four hundred-mesh copper grids (Ted Pella Co.) were mounted upon the floating film, and the entire

preparation picked up from the water by means of cut Parafilm. After complete drying, the coated grids were used immediately or stored for no longer than 48 hours in a clean Petri dish placed under vacuum in a desiccator. Optionally, grids were overlaid with an evaporated monolayer of graphitic carbon (Ladd Research Industries, Inc.) by the method of Bradley (1) and used during the day of coating.

Biological Wetting Agents - Powdered bacitracin (Sigma Chemical Co.), bovine serum albumin (Sigma Chemical Co.), and sucrose crystals (Mallinckrodt, Inc.) were dissolved in filtered distilled water to give concentrations of 500 $\mu\text{g/ml}$, 0.01%, and 0.2%, respectively. Each freshly-prepared spreading agent was mixed with an equal volume of negative stain immediately prior to use. Weighed amounts of spreading base were also dissolved directly in negative stain to achieve the desired concentration. Bacitracin and bovine serum albumin powders were stored in a desiccator at 4 C and sucrose was kept at room temperature.

Negative Staining Procedure - Negative staining was performed by the method of Huxley and Zubay (10). Plastic filmed grids, with or without carbon coating, were clamped on edge with a pair of positive-pressure fine-point forceps and the forceps rested upon a level surface with the support side of the grid facing upwards. Using a clean Pasteur pipette, a small droplet of specimen (approximately 5 μl) was placed upon the grid surface in a bead barely extending from one edge of the grid to the other. The specimen was allowed to adsorb to the grid film for an appropriate amount of time (5 seconds to 1 minute, 15 seconds average), and the excess fluid removed at the grid edge with a small

cut piece of Whatman #1 filter paper. An equivalent amount of negative stain was immediately applied to the grid surface for several seconds, and the excess fluid removed as before, leaving a thin residual film to dry upon the grid. The preparation was allowed to dry thoroughly for 5 minutes before examining it in a Philips 300 electron microscope operated at 80 kV with double condenser illumination and a 30 μ m gold foil objective aperture in place.

Grid Examination and Photography - Grids were scanned initially at instrumental magnifications of 20,000X and 60,000X, for 5 to 7 minutes, respectively, to determine the presence of virus. Methanol precipitates were negatively stained and scanned under the electron microscope as described, and the virus particles contained upon each of 10 randomly selected central grid squares quantitated numerically. Original unprecipitated lysates were examined in an identical manner. Representative virus particles from clarified lysates were photographed at optical magnifications of 60,000X or 80,000X as single shots or 3 to 5 shot focal series, using Kodak Estar Thick Base or SO-163 Electron Microscope Film (Eastman Kodak Co.), and a 1 second exposure time, to minimize specimen drift. Negatives were developed in Kodak D-19 Developer, fixed in Kodak Rapid Acid Fixer, and washed in running water for 20 to 30 minutes. Subsequently, they were briefly immersed in Kodak Photo-Flo and dried for 30 minutes in a hot air dryer. Positive prints were made by enlarging negatives to 160,000X with tungsten bulb point light source enlarger (Simmons Omega Inc., Type M6) and printing them upon graded (1 through 5) medium weight Kodak Kodabrome RC paper, glossy (F) surface.

Table 1. Viruses examined by negative contrast electron microscopy

Virus	Strain or Designation	Contributor	Cell Culture
<u>Control Viruses</u>			
Pseudocowpox	MSU isolate ¹	MSU	Bovine fetal kidney (BFK)
Bovine adenovirus, type 3	WBRI ²	CVL	BFK
Parainfluenza, type 3	MSU isolate	MSU	BFK
Feline rhino-tracheitis	MSU isolate	MSU	Feline kidney (CRFK ³)
Feline calici-virus	MSU isolate	MSU	CRFK
<u>Other Viruses</u>			
Vesicular stomatitis	Indiana	ATCC ⁴	BFK
Swine polioen-cephalomyelitis	O3b	ATCC	Primary porcine fetal kidney (PPFK)
Transmissible gastroenteritis	Purdue	ATCC	PPFK
Porcine parvovirus	---	NADC ⁵	PPFK
Reovirus, type 3	Abney	ATCC	HeLa
Mouse polyoma	LID-1	ATCC	Primary mouse kidney
Equine influenza	A/Equine 1/ Prague/1/56	ATCC	Chicken embryo (allantoic fluid)

¹Clinical Virology Laboratory, Michigan State University.

²Mr. P. Stuart, Central Veterinary Laboratory, Ministry of Agriculture, Fisheries, and Food, New Haw, Weybridge, Surrey, England.

³Crandell-Rees feline kidney.

⁴American Type Culture Collection, Rockville, Maryland.

⁵National Animal Disease Center, Ames, Iowa.

RESULTS

Type of Negative Stain - Of the five negative stains studied, phosphotungstic acid (PTA) routinely produced high quality images which exhibited fine granularity, excellent three-dimensional detail, and good preservation of virus structure. PTA consistently spread into a thin uniform stain deposit over fresh hydrophilic support films without the addition of a biological wetting agent when clarified virus preparations were examined. Two disadvantages associated with PTA staining were the tendency of the negative stain to enclose virus particles within an overly dense halo and the occasional membrane rupture which occurred with enveloped viruses. Sodium silicotungstic acid and cobaltous tungstate compared favorably with PTA in overall image quality, but both negative stains always required the presence of a spreading base to form an acceptable stain bed. Ammonium molybdate produced images possessing softer contrast and predominantly surface detail, and was also difficult to spread into a thin stain bed without the use of a spreading agent. Slightly better membrane preservation was observed for enveloped viruses when ammonium molybdate was employed. Uranyl acetate gave images with the finest granularity and the highest contrast, but these had a flatter two-dimensional appearance than seen with the other negative stains. Other disadvantages associated with uranyl acetate were its poor spreading ability and its incompatibility with proteins often found in clarified lysates. Enveloped viruses stained with uranyl acetate demonstrated primarily surface detail and a more uniform shape.

Negative Stain Concentration - One per cent PTA deposited a stain bed which was minimally thin yet provided excellent detail and contrast of virus structure. A concentration of one-half per cent ammonium molybdate was found also to be suitable for the negative staining of enveloped viruses.

Negative Stain pH - One per cent PTA, adjusted to a slightly acid pH (6.5) with 5N KOH, was found to provide the best general negative stain detail and contrast for three of the control virus types studied (Figures 1 through 7), including the complex DNA virus (pseudocowpox virus), the nonenveloped DNA virus (bovine adenovirus, type 3), and the enveloped helical RNA virus (parainfluenza virus, type 3). Although the same negative staining conditions were used for the enveloped DNA virus (feline rhinotracheitis virus) with good results (Figures 8 and 9), a neutral pH of 7.0 produced optimal negative staining, particularly in regard to structural preservation. One per cent PTA, pH 5.0, was used to contrast certain nonenveloped RNA viruses (feline calicivirus), although one per cent PTA at pH 6.5 proved equally good for others (swine polioencephalomyelitis virus, Figure 10). One-half per cent ammonium molybdate, pH 7.0, was successfully applied to enveloped helical RNA and enveloped DNA viruses. Standardized negative staining conditions (one per cent PTA, pH 6.5) were extrapolated to seven additional animal viruses (Figures 11 through 13).

Plastic Support Films - Parlodion films cast upon precleaned glass slides from 0.25 to 0.5% solutions in amyl acetate for photography and routine examination of lysates, respectively, afforded the best

type of plastic support substrate. Parlodion films offered the advantages of easy preparation, a fine-grained, smooth background for negative staining, good hydrophilicity, and avid adsorption of virus particles. Mechanical weakness under the electron beam was a drawback, and frequent electrostatic charge disruption of Parlodion films was encountered. Butvar B-98 films fabricated from 0.25% solutions in ethylene dichloride possessed exceptional strength, adhered well to the grid bars, and gave fairly smooth surface spreading of negative stain preparations when a wetting agent was present. Film preparation was tedious. Formvar films cast from similar solutions were also endowed with good mechanical strength but wetting agents were required for adequate spreading of samples and negative stain over support surfaces. Although Formvar film fabrication was easy, support surfaces were rough, uneven, and often patterned. Failure to detach from casting surfaces was also a common problem. Both Butvar B-98 and Formvar films adsorbed virus less readily (3 to 5 times) than Parlodion supports. Carbon strengthening was optional for all three film types.

Biological Wetting Agents - Bacitracin was found to be the spreading base of choice, offering the advantages of compatibility with all five negative stains studied, nonreactivity with specimen molecules, and small, nonvisualizable molecules. When used with PTA, even spreading of stain occurred, and the characteristic densely contrasting halo of negative stain commonly associated with PTA was reduced in size. Excellent spreading also was observed when bacitracin was used as a base for the remaining four negative stains. Bovine serum albumin (BSA) offered a fair alternative to bacitracin, but significant

increases in background granularity nearly always occurred due to its molecular size, and denaturation of the protein during solubilization or in the presence of negative stain was a common event. The denaturation of BSA was most pronounced when it was used as a base for uranyl acetate. Sucrose was compatible as a base for all five negative stains, but at the minimal concentration required for stain spreading, coating of the virus particles usually occurred, with a reduction in detail and contrast.

Methanol Concentration - A comparison of unprecipitated original lysates with methanol precipitates of the same materials prediluted 100-fold revealed approximately 80 to 90 per cent recovery of virus for all control morphological virus types, based on direct numerical counting. Morphologically recognizable virus particles were present, together with partially or wholly degraded virions. We have successfully applied this procedure with several modifications to the concentration of calf scours coronavirus particles from 20 per cent fecal homogenates. The thorough mixture of dry KCl with the crude suspension prior to initial centrifugation to achieve a final concentration of 1M has proved beneficial in inhibiting protein-lipid and protein-protein electrostatic interactions which can result in the trapping of large clumps of virus mixed with cellular debris. Similarly, resuspension of methanol pellets in dilute neutral phosphate buffer appeared to elute the virus particles from each other and from contaminating host material. Unwanted debris also obscured virus particles and caused support film disruption due to uncontrollable heating and electrostatic charge buildup which occurs during electron microscopy. Further removal of contaminating cellular debris was

achieved for nonenveloped viruses by the rapid extraction of resuspended methanol pellets with an equal volume of cold fluorocarbon (Genesolv-D) as a final step. End step filtration of suspensions through small pore membrane filters produced a similar result for all virus types, although some loss in detectable numbers occurred.

DISCUSSION

We have routinely employed one per cent phosphotungstic acid (PTA) at slightly acid or neutral pH values for the negative staining of viruses commonly encountered in veterinary medicine. PTA stain gave an excellent quality image which exhibited fine granularity and detail, a three-dimensional appearance, and good preservation of structure, in agreement with Haschemeyer and Meyers (7). When hydrophilic support films and crude virus preparations were used, PTA spread well over the support surface without the addition of any biological wetting agent. A concentration of one per cent PTA resulted in a thin stain bed which enhanced the fine detail and contrast of virus structure. Lower concentrations of PTA (e.g., 0.5%) produced a low contrast image and often failed to project observable detail or to cover the support film adequately. Similarly, higher negative stain concentrations (e.g., 2 or 4%) tended to over-embed virus particles and to reduce contrast and obscure detail. A slightly acid stain pH appeared to result in a good balance between surface staining and penetration of stain into the interior of the virion. Markedly acid stain pH values gave predominantly surface staining, and alkaline pH's increased entry of negative stain into the virus. Extremes of stain pH produced a greater proportion of disrupted virus particles. PTA was stable for long periods when

protected from CO₂ dissolution, compatible with low concentrations of media buffers and cellular debris, and cheaply and commercially available. PTA has the known tendency to disrupt membranes by exerting electrostatic charge stress (9). The use of neutral PTA or an osmotically and electrostatically gentle negative stain such as ammonium molybdate (13) proved to be a reasonable alternative for negatively staining enveloped viruses.

Because of their hydrophilicity, Parlodion support films gave good PTA stain spreading without the use of a wetting agent, when they were freshly prepared and protected from attack by moisture. Parlodion films were easily prepared and rendered a smooth, fine-grained background in negative stain images. Virus adsorption to Parlodion was noticeably greater than with Butvar B-98 and Formvar films, so that consistently more virus particles were recovered. This represents an important consideration for clinical specimens where virus titers are limited. Although a comparative lack of mechanical strength remained a disadvantage, Parlodion films could be stabilized by carbon strengthening and/or by slowly tracing the grid square edges with the electron beam while increasing the beam current and the intensity of illumination stepwise to approach the point of crossover. Butvar B-98 support films did not prove to be as wettable as previously reported (14).

As previously observed (6), bacitracin afforded a biological spreading agent which was superior to bovine serum albumin or sucrose. A uniform stain bed resulted when bacitracin was used as a wetting base; no denaturation or visualization of the protein occurred during its preparation or in the presence of any of the five negative stains

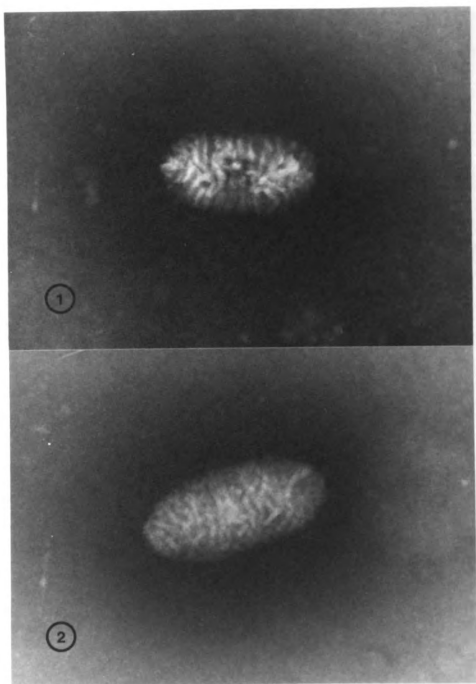
studied, and fine details of virus structure were unobscured. Bactracin reduced the obscuring coalescence of PTA around virus particles.

Methanol precipitation of all major morphological virus types from dilute distilled water lysates and 20 per cent fecal homogenates provided an excellent means for concentrating virus 80- to 90-fold. Negative contrast electron microscopy of clarified precipitates showed typical virions observed singly or in large aggregates.

In summary, the use of one per cent PTA, pH 6.5, with optionally carboned Parlodion support films to examine crude virus preparations directly or preferably after methanol concentration offers the best means for recovering virus and for observing virus morphology as an aid to the diagnosis of viral disease.

Figure 1. Pseudocowpox particle demonstrating a typical ovoid shape with a complex surface pattern of striated ridges.

Figure 2. Pseudocowpox particle surrounded by its thin unspiked membrane.



Figures 1 and 2

Figure 3. Naked isometric adenovirus virions demonstrating round surface capsomers arranged in icosahedral symmetry. Both complete and incomplete particles are present.

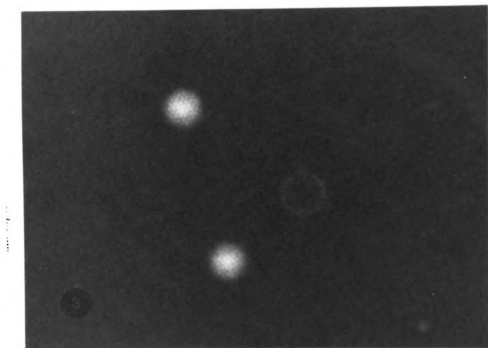
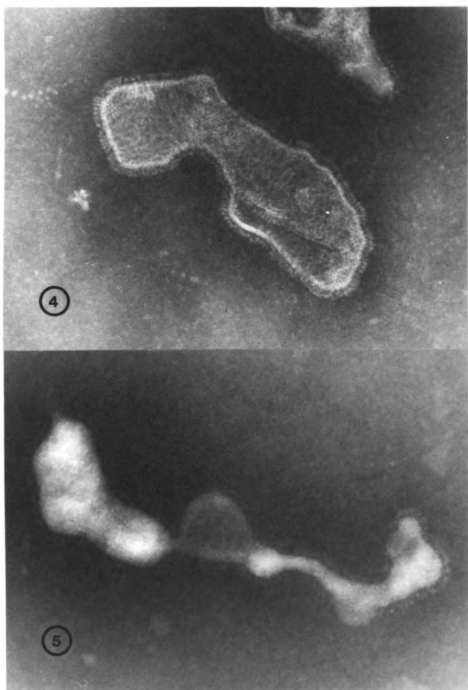


Figure 3

Figure 4. Four paramyxovirus structures are observable by PTA embedding (Figures 4 through 7). Note that all enveloped particles (Figures 4 through 6) possess a well-defined outer envelope covered with a regular fringe of short globular spikes. Figure 4 depicts a whole paramyxovirus virion exhibiting partial negative stain penetration into the envelope interior to reveal the internal nucleocapsid.

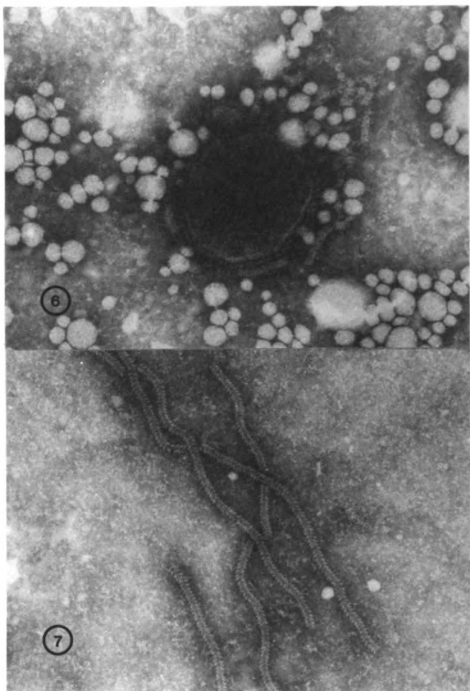
Figure 5. Intact enveloped virus demonstrating entirely surface staining.



Figures 4 and 5

Figure 6. Incomplete destruction of the paramyxoviral envelope has occurred, releasing the internal helical nucleoprotein component and allowing entry of negative stain into the envelope to outline the remaining helix.

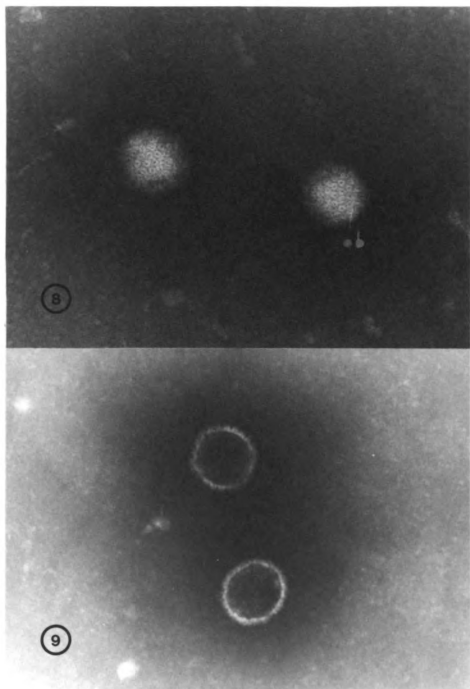
Figure 7. Occasional free paramyxovirus helices demonstrating a characteristic width, a central hole, and serrations of regular periodicity.



Figures 6 and 7

Figure 8. Two morphological particle types seen with feline rhinotracheitis virus (Figures 8 and 9). Mature naked virions are composed of elongated hollow prismatic capsomers roughly hexagonal in shape, in icosahedral array.

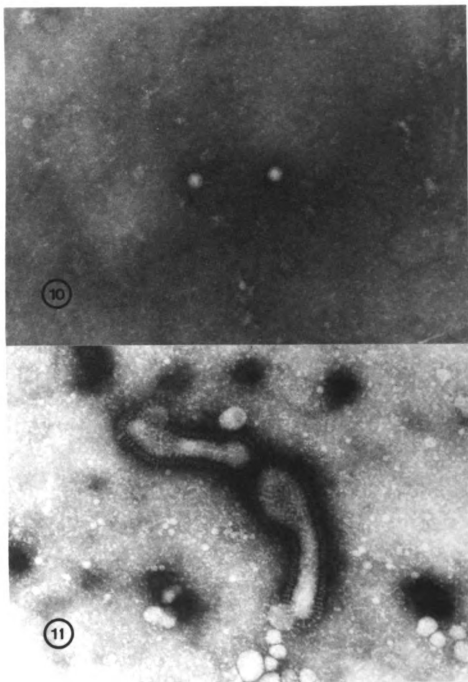
Figure 9. Empty capsides of herpesvirus with dark centers and well-delineated outer protein shells are seen. Note side view of capsomer structure on upper particle.



Figures 8 and 9

Figure 10. Icosahedral particles with little or no observable surface detail are characteristic of porcine polioencephalomyelitis virus (picornavirus).

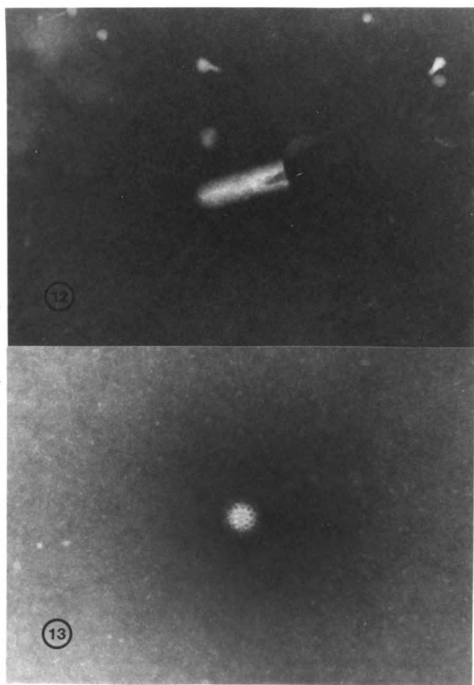
Figure 11. Pleomorphic equine influenza virus virions showing a well-defined outer envelope which is fringed with long rectangular peplomers. The envelope is resistant to negative stain penetration.



Figures 10 and 11

Figure 12. Bullet-shaped virion of vesicular stomatitis virus with its thin, closely-adherent, finely-spiked envelope.

Figure 13. Reovirus particle showing its round double-shelled capsid and surface indentations.



Figures 12 and 13

1. The first part of the report deals with the general situation of the country and the position of the various groups. It is a very good summary of the situation and is well written. The second part of the report deals with the specific details of the situation and is also well written. The third part of the report deals with the recommendations and is also well written. The fourth part of the report deals with the conclusions and is also well written. The fifth part of the report deals with the appendix and is also well written. The sixth part of the report deals with the bibliography and is also well written. The seventh part of the report deals with the index and is also well written. The eighth part of the report deals with the list of figures and is also well written. The ninth part of the report deals with the list of tables and is also well written. The tenth part of the report deals with the list of references and is also well written. The eleventh part of the report deals with the list of abbreviations and is also well written. The twelfth part of the report deals with the list of symbols and is also well written. The thirteenth part of the report deals with the list of units and is also well written. The fourteenth part of the report deals with the list of formulas and is also well written. The fifteenth part of the report deals with the list of equations and is also well written. The sixteenth part of the report deals with the list of diagrams and is also well written. The seventeenth part of the report deals with the list of maps and is also well written. The eighteenth part of the report deals with the list of photographs and is also well written. The nineteenth part of the report deals with the list of illustrations and is also well written. The twentieth part of the report deals with the list of figures and is also well written.

LITERATURE CITED

1. Bradley, D. E. 1965. The preparation of specimen support films, pp. 58-74. In D. Kay (ed.), *Techniques for Electron Microscopy*, Blackwell Scientific Publications, Oxford.
2. Davis, C. M., G. Musil, and J. A. Trochet. 1970. Electron microscopy for the rapid diagnosis of pseudocowpox and milker's nodule. *Am. J. Vet. Res.* 31:1497-1503.
3. England, J. J., C. S. Frye, and E. A. Enright. 1976. Negative contrast electron microscopic diagnosis of viruses of neonatal calf diarrhea. *Cornell Vet.* 66:172-182.
4. England, J. J., R. M. Barratt, E. A. Enright, and C. S. Frye. 1976. Diagnosis of viral respiratory infections by direct electron microscopic examination of nasal swabs, pp. 377-384. In *Proceedings of the 19th Annual Meeting: The American Association of Veterinary Laboratory Diagnosticians*. American Association of Veterinary Laboratory Diagnosticians, 6101 Mineral Point Road, Madison, Wisconsin.
5. Goodheart, C. R., G. R. Armstrong, D. V. Ablashi, G. Pearson, and T. W. Orr. 1974. Concentration of oncogenic herpesviruses by methyl alcohol precipitation. *Appl. Microbiol.* 27:988-990.
6. Gregory, D. W., and B. J. S. Pirie. 1973. Wetting agents for biological electron microscopy. I. General considerations and negative staining. *J. Microsc. (Oxford)* 99:251-265.
7. Haschemeyer, R. H., and R. J. Meyers. 1972. Negative staining, pp. 100-147. In M. A. Hayat (ed.), *Principles and Techniques of Electron Microscopy: Biological Applications*, Volume 2. Van Nostrand Reinhold Co., New York.
8. Hayat, M. A. 1970. Support films, pp. 323-332. In M. A. Hayat (ed.), *Principles and Techniques of Electron Microscopy: Biological Applications*, Volume 1. Van Nostrand Reinhold Co., New York.
9. Horne, R. W. 1967. Electron microscopy of isolated virus particles and their components, pp. 521-574. In K. Maramorosch and H. Koprowski (eds.), *Methods in Virology*, Volume 3. Academic Press, Inc., New York.
10. Huxley, H. E., and G. Zubay. 1960. Electron microscope observations on the structure of microsomal particles from *Escherichia coli*. *J. Mol. Biol.* 2:10-18.
11. McFerran, J. B., J. K. Clarke, and W. L. Curran. 1971. The application of negative contrast electron microscopy to routine veterinary virus diagnosis. *Res. Vet. Sci.* 12:253-257.

12. Marsolais, G., L. Berthiaume, E. DiFranco, and P. Marois. 1971. Rapid diagnosis by electron microscopy of avian coronavirus infection. *Can. J. Comp. Med.* 35:285-288.
13. Munn, E. A. 1968. On the structure of mitochondria and the value of ammonium molybdate as a negative stain for osmotically sensitive structures. *J. Ultrastruct. Res.* 25:362-380.
14. Oliver, R. M. 1973. Negative stain electron microscopy of protein macromolecules, pp. 616-672. In C. H. W. Hirs and S. N. Timasheff (eds.), *Methods in Enzymology: Enzyme Structure*, Volume 21D. Academic Press, Inc., New York.
15. Scott, A. C., P. H. Lamont, M. S. Chapman, and J. D. J. Harding. 1973. Electron microscopy in the rapid diagnosis of inclusion-body rhinitis of pigs. *Vet. Rec.* 93:127-128.
16. Spradbrow, P. B., and J. Francis. 1969. Electron microscopy as an aid to the rapid identification of animal viruses. *Vet. Rec.* 84:244-246.
17. Van Kammen, A., and P. B. Spradbrow. 1976. Rapid diagnosis of some avian virus diseases. *Avian Dis.* 20:748-751.

MICHIGAN STATE UNIV. LIBRARIES



31293103169011