ACROLEIN - A FINE STRUCTURE FIXATIVE FOR VIRAL CYTOCHEMISTRY

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The simplest architectural arrangement favored for infectious virus particles involves the encapsulation of a core of nucleic acid (either DNA or RNA) in a coat or capsid of antigenic protein. For a fine structure fixative to be a suitable candidate for viral cytochemistry, it must allow at least the identification and localization of these two fundamental moities. Such a fixative should preserve the antigenicity of viral proteins and allow their specific demonstration with labeled antibody conjugates (3, 24). The fixative should also preserve viral nucleic acids so that they may be identified and located by means of simple fluorochrome procedures (1, 15). A satisfactory performance includes the ability to carry out the relevant enzyme digestion tests necessary to establish specifically the nucleic acids identified. In addition, a degree of fine structural preservation of both virus particles and cell organelles should be attained so that examination of ultrathin sections of virus-infected cells in the electron microscope becomes a meaningful endeavor.

Most viral antigens are well fixed by acetone (4), but this agent is a totally inadequate fixative for viral or cellular nucleic acids or for fine structure. Alcoholic fixatives such as Carnoy's fluid (60 per cent ethanol, 30 per cent chloroform, 10 per cent acetic acid), ethanol, ether-alcohol, and acetic alcohol allow a brilliant demonstration of both cellular and viral nucleic acids with acridine orange staining techniques (1, 16, 14). Both proteases and nucleases are fully active on such fixed material, enabling a confident identification of nucleic acid type. However, these fixatives do not preserve viral antigens and, although useful for demonstrating nucleic acids in purified virus preparations (30), are totally inadequate preservatives of cellular fine structure. Osmium tetroxide and potassium permanganate are excellent fine structural fixatives for electron microscopy, and enzyme digestion tests can be carried out on permanganate-fixed viruses (7, 8). However, although a few bacterial antigens appear to survive osmium tetroxide fixation (27), both the aforementioned fixatives denature viral antigens and are also quite inadequate for routine fluorescence microscopy.

Formol (29, 10) and formol-calcium (24, 20, 10) have been the most successful fixatives, to date, for microcytochemistry and, in combination with water-soluble embedding materials, lead to identifiable electron images (2). However, cell fine structure is not well preserved routinely, and the results in the fluorescence microscope are not impressive. Use of these fixatives has led to an elegant demonstration of viral antigens in the electron microscope with ferritin-labeled antibodies (26, 24, 20), but postfixation with osmium tetroxide is necessary for a full revelation of cellular fine structure, and staining with specific antibody conjugates cannot, therefore, be carried out directly on ultrathin sections.

In Table I the performances of a number of the so called "conventional" fixatives, as assayed from observations in both the fluorescence and electron microscopes, are summarized.

Interest in acrolein as a fine structure fixative for electron microscopy was stressed by Luft (12) who was particularly impressed by its rapid penetration of tissue components in contrast to osmium tetroxide. Feder (9) pointed out the superiority of highly polar organic solvents,

such as methanol, as dehydrating agents after acrolein fixation, and Watson and Aldridge (31) stressed acrolein's importance as an organic fixative which could be used in conjunction with specific heavy metal stains.

This report presents some observations obtained with acrolein as a preserver of viral antigens and nucleic acids at the cellular level, and of virus architecture at the fine structural level. This fixative appears to be one of the most adequate for viral cytochemistry yet encountered.

TABLE I

Performance of Fixatives for Viral Microcytochemistry

Fixative	Fluorescence Microscopy: Viral antigen	Fluorescence Microscopy: Viral and cellular nucleic acid: Performance	Fluorescence Microscopy: Viral and cellular nucleic acid: Enzyme digestion control	Electron Microscopy: Viral antigen	Electron Microscopy: Fine structure: Viral nucleic acid	Electron Microscopy: Fine structure: Virus particles	Electron Microscopy: Fine structure: Virus-cell system
Acetone	Excellent	Weak	Weak	—	—	—	—
Carnoy's fluid	—	Excellent	Excellent	—	—	—	—
Acetic-alcohol	—	Excellent	Excellent		Adequate		Adequate for cellular fine structure
Osmic acid	—	—	—	—	Excellent − enzyme	Excellent	Excellent
Potassium permanganate	—	—	—	—	Adequate + enzyme	Excellent	Excellent
Formol	Variable	Poor	Poor	Good	Adequate + enzyme	Adequate	Only adequate when post-fixed with osmic acid

MATERIALS AND METHODS

Tissue Culture and Viruses

Trypsin-dispersed epithelial cells from either rhesus or green monkey kidneys were grown in 16-oz bottles or on 11 × 22 mm coverslips in Leighton tubes, using methods and media previously described (18). The following stock viruses were available in the Department and were used: poliovirus, type 2 (MEF1); reovirus, type 1 (#716); adenovirus type 3 (Rubin), and the simian papovavirus, SV40, that had been isolated from Sabin type 3 oral vaccine (28). Conditions for inoculation, adsorption, growth, and harvest are shown in Table II.

Adenovirus, poliovirus, and reovirus were grown in rhesus monkey cells, SV40 in green monkey cells. At harvest, an advanced specific cytopathic effect could be observed in the light microscope with each viral agent.

Electron Microscopy

Infected and non-infected tissue culture monolayers in 16-oz bottles were fixed *in situ* with 1 per cent acrolein[1] in phosphate-buffered saline pH (7.2) for 10 minutes at room temperature, dehydrated rapidly in methanol series, and impregnated for 2 hours in a mixture of epoxy resin, CIBA 502 and propylene oxide (1:1). The cells were then scraped from the glass surface and embedded as a pellet in fresh resin containing DMP 30[2] as accelerator. Control preparations

[1] All manipulations with acrolein should be carried out in a fume hood or well ventilated area.

[2] 2,4,6-Tri(dimethylaminomethyl)phenol; Rohm & Haas Co., Philadelphia, Pa.

after cold fixation in 1 per cent buffered osmium tetroxide (21) were treated in the same manner. Ultrathin sections were cut on a Siroflex ultramicrotome equipped with glass knives, mounted on carbon-coated copper grids, and examined in the Siemens Elmiskop I at magnifications ranging from 5000 to 20,000.

Fluorescence Microscopy

Detection of Viral Antigen

Infected and uninfected coverslip cultures were harvested and fixed immediately at room temperature in 1 per cent buffered acrolein for 10 minutes, rinsed in 3 changes of phosphate-buffered saline, and air dried. Companion control preparations were air dried, fixed in acetone for 10 minutes, and dried again. Coverslips were incubated with 2 drops of the fluorescein-labeled antibody conjugates in a moist chamber at 37°C for 30 minutes, washed in buffer, and examined in the fluorescence microscope. In all cases, development of virus-specific fluorescence was completely inhibited by treatment with unconjugated immune sera prior to staining with the labeled conjugates. Uninfected control preparations did not develop any detectable fluorescence when stained with the fluorescent conjugates. The following laboratory stock antisera were made available:

SV40 rabbit antiserum (neutralizing antibody titer > 1/10,000); poliovirus monkey antiserum (neutralizing antibody titer 1/2,500); reovirus, monkey antiserum (neutralizing titer 1/27,000); adenovirus rabbit antiserum (neutralizing titer 1/320). Methods for preparing the globulin fractions and their conjugation with fluorescein label have been described in detail (13).

Detection of Viral Antigen in Thick Sections

Thick sections were cut from the acrolein-fixed tissue blocks infected with reovirus and SV40 prepared for electron microscopy (see above). Sections were picked up on standard microscope slides and dried. They were stained without removal of the resin by the indirect fluorescent antibody procedure (4), using the unlabeled antibody globulin described above and a fluorescein-labeled anti-monkey rabbit globulin.

TABLE II

Virus Inoculation, Growth, and Harvest

Virus	Leighton tube culture	16 oz. Bottle culture	Adsorption time	Harvest time
Adenovirus types 3 and 7	0.1 ml Suspension containing $10^{3.5}$ ID_{50}/ml	1 ml Suspension containing $10^{3.5}$ ID_{50}/ml	2 hrs.	72 hrs. postinoculation
Poliovirus type 2	0.1 ml Suspension containt 10^{7} PFU/ml	1 ml Suspension containing 10^{7} PFU/ml	1 hr.	24 hrs. postinoculation
Reovirus type 1	0.1 ml Suspension containing $10^{6.5}$ PFU/ml	1 ml Suspension containing $10^{6.5}$ PFU/ml	2 hrs.	72 hrs. postinoculation
SV40 papovavirus	0.1 ml Suspension containing 10^{7} PFU/ml	1 ml Suspension containing $10^{7.5}$ PFU/ml	4 hrs.	96 hrs. postinoculation

Detection of Viral Nucleic Acids

Infected and uninfected coverslips were harvested and placed immediately in a 1 per cent solution of acrolein in methanol at room temperature for 10 minutes. After rapid hydration in a methanol series the coverslips were stained with acridine orange by the standard procedure (13, 17). Control companion preparations were fixed in Carnoy's fluid and processed as described above. Enzyme digestion tests were carried out on similar acrolein and Carnoy-fixed preparations prior to acridine orange staining (see 17 for technical details.)

RESULTS

Examination of acrolein-fixed infected tissue culture monolayers in the fluorescence microscope

revealed that the degree of preservation of all the viral antigens tested was excellent after acrolein fixation (Figs. 1 and 2) and in no way inferior to the results obtained after fixation with acetone, the standard fixative for antigens of animal viruses. Although general staining of intracellular nucleic acids after acrolein fixation was not quite so bright as in companion Carnoy-fixed cultures, viral inclusions stained brilliantly (Figs. 3 and 4) and specific enzyme digestion tests could be carried out in a convincing manner. The DNA-containing viruses (adenovirus, SV40) required the use of a protease before the nucleic acid became susceptible to DNase. This susceptibility pattern is also characteristic of Carnoy-fixed DNA animal viruses (1, 17).

In ultrathin sections of acrolein-fixed, epoxy-embedded material the degree of preservation of virus architecture was often quite striking, particularly in the case of those viruses which form intracellular crystalline arrays (Figs. 5 and 6). Reovirus which yields an abundance of both complete and empty forms in standard osmium-fixed material (23) revealed the same qualities after acrolein fixation (Fig. 6).

Coverslip preparations of cells infected with adenovirus, SV40, reovirus, and poliovirus have also been taken through each stage in the standard dehydrating and embedding procedure for electron microscopy, except the final polymerization. In each case, even when coverslips had soaked for several hours in the resin and its accelerator, the preparations stained brilliantly with their specific fluorescein-labeled antibody conjugates. Thick sections cut from acrolein-fixed blocks infected with reovirus and SV40 revealed positive findings of viral antigen in the fluorescence microscope after staining with the indirect fluorescent antibody procedure. These preliminary observations indicate that certain viral antigens survive the complete dehydrating and embedding procedure and that direct loca-

FIGURE 1

Monkey kidney cells infected with adenovirus type 3 fixed in 1 per cent buffered acrolein and passed through epoxy resin and accelerator before staining. Fluorescent antibody technique. Blocks of bright staining antigen are located in the nuclei of infected cells.

FIGURE 2

Monkey kidney cells infected with reovirus type 1 fixed in 1 per cent buffered acrolein and passed through epoxy resin and accelerator before staining. Fluorescent antibody technique. The cytoplasm of infected cells is filled with antigen.

FIGURE 3

Monkey kidney cells infected with adenovirus type 3 fixed in 1 per cent alcoholic acrolein. Acridine orange technique. Inclusions of yellow green DNA (*I*) are present in the nuclei of infected cells. (Black and white reproduction from color original).

FIGURE 4

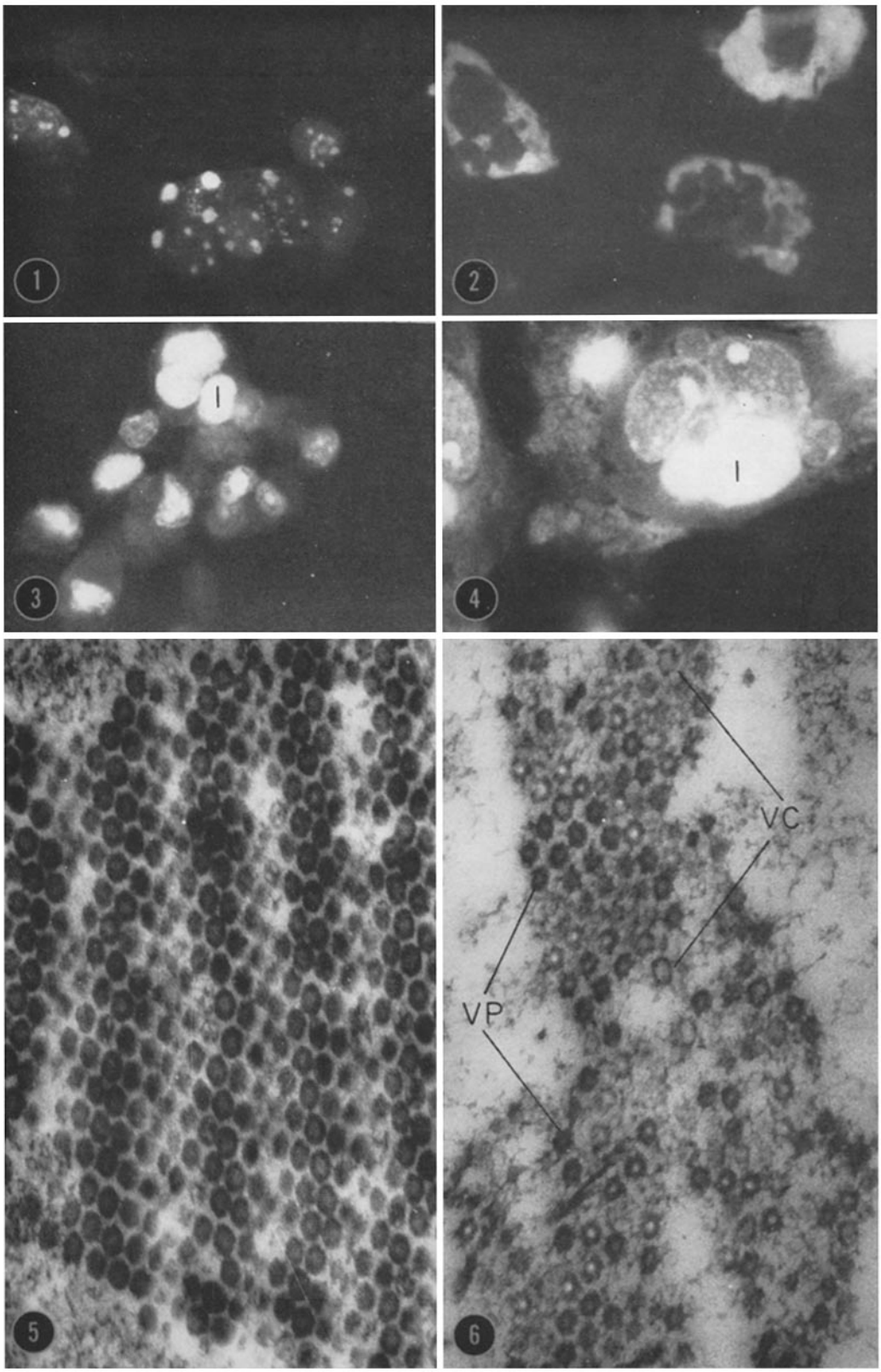
Monkey kidney cells infected with reovirus type I fixed in 1 per cent alcoholic acrolein. Acridine orange technique. Cytoplasmic inclusions of flame red viral RNA (*I*) are present. (Black and white reproduction from color original).

FIGURE 5

Ultrathin section through the nucleus of a monkey kidney cell infected with adenovirus type 3. Acrolein fixation. Arrays of hexagonal-shaped virus particles are visible. Magnification, 62,000.

FIGURE 6

Ultrathin section through the cytoplasm of a monkey kidney cell infected with reovirus type I. Acrolein fixation. Close packed virus particles (*VP*) and many empty forms (*VC*) are shown. Magnification, 62,000.


1
2
I
3
I
4
5
VC
VP
6

tion of viral antigens and nucleic acids at the fine structural level, using ferritin (26) or heavy metal labels such as mercury (22), will certainly be possible.

DISCUSSION

The observations reported here indicate that acrolein is a competent fixative for animal virus antigens and cellular and viral nucleic acids. In particular, the degree of preservation of viral antigens is remarkable. It is relevant to note here that Van Duijn has reported the usefulness of acrolein in demonstrating cellular proteins (5) and that Kamen (11) was able to obtain efficient immunization of animals by using acroleinized virus preparations which had been stored for a number of years.

We have observed at the fine structural level routinely adequate preservation of cellular organelles and image detail of satisfactory electron contrast. In many preparations, particularly with the larger viruses (*e.g.*, adenovirus and reovirus), the preservation of virus architecture is often superior to that achieved in companion preparations fixed in osmium tetroxide.

To date, the most successful attempts at locating viral antigens in infected cells with electron microscopy have employed the use of ferritin-labeled conjugates introduced prior to polymerization of materials processed for ultramicrotomy (24, 20). Similar techniques have been used to demonstrate cellular antigens (6).

The positive finding that certain viral antigens can survive acrolein fixation and the complete dehydrating and embedding procedures employed for electron microscopy will be an aid in the development of a direct use of antibody under the electron microscope. Such a procedure would be not only an effective tool in identifying new viral agents within the cell, but also a means of discriminating among the host of "virus-like particles" which can be detected in many embedded tissues (see, for example, Ziegel, 32) and which are all possible candidates as oncogenic viruses.

Recently there have been a number of reports on the success of other aldehydes as both histochemical and fine structure fixatives. In particular, the performance of glutaraldehyde seems most promising, particularly for the demonstration of intracellular enzymes (25). We have carried out some preliminary investigations comparing 1 per cent glutaraldehyde in Millonig's buffer (19) and acrolein. Both fixatives gave an equally satisfactory demonstration of cellular and viral nucleic acids in the fluorescence microscope. However, the performance of acrolein as a preserver of viral antigens was markedly superior.

A preliminary report on these findings was presented by the authors at the 5th International Congress of Electron Microscopy, abstract X-12, 1962. At the same meeting the importance of acrolein as a fixative was also stressed in papers by Bernhard and Leduc (abstract L-4) and by Granboulan and Granboulan (abstract PP-3).

The continuing support of Dr. J. L. Melnick is gratefully acknowledged.

Aided by grants from the National Foundation and from the National Cancer Institute (Grant C-4600), and by a training grant, 2E-74, from the National Institute of Allergy and Infectious Diseases.

Received for publication, January 25, 1963.

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