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Preface

The favorable reception given to Volume 1 of this serial publication provides sufficient justification for continuing the policies on which it was based. Accordingly, the Editors have interpreted "experimental pathology" in its widest sense, and have encouraged contributors to emphasize and explain the basic scientific aspects of their subjects in terms suitable for workers in other biomedical disciplines. As in the previous volume, topics for review have been selected from areas that are currently of special significance or in which unusual progress has been made. These criteria have also been used in planning for the future.

Once again, the Editors are much indebted to the members of the Editorial Advisory Board for their valuable help and support.

October, 1963

G. W. RICHTER
M. A. EPSTEIN

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The Nucleic Acids of Viruses as Revealed by Their Reactions with Fluorochrome Acridine Orange

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I. Introduction

At the molecular level of research in viral cytopathology the fluorochrome, acridine orange (AO), is of special importance among the vital dyes because it has the dual capacity to act not only as a vital stain which can be incorporated into the virus particles during their intracellular replication cycle (Crowther and Melnick, 1961; Mayor and Diwan, 1961), but also as a sensitive cytochemical indicator for the detection and identification of nucleic acids (Anderson *et al.*, 1959; Pollard and Starr, 1962). In addition the fluorochrome is an effective agent of viral photodynamic inactivation (Chessin, 1960; Crowther and Melnick, 1961; Mayor *et al.*, 1962b) and a member of the aminoacridines, a proved mutagenic group of compounds (Orgel and Brenner, 1961; Brenner *et al.*, 1961). Thus the acridine orange molecule (Fig. 1) has the possibility of being an extremely valuable tool in any integrated cytochemical and biological study of the basic mechanisms of viral replication.

This review focuses attention on the diversity of this fluorochrome as an accessory to and "interloper" in the virus-host cell relationship both during the replication process and after the mature virus particles are released. An attempt will be made to draw together from the relevant literature some of the significant findings in this rapidly moving area of research. In general, there will be a concentration on experiments performed and results obtained in the author's laboratory with a number of viruses and animal virus-host cell systems. The investigator can acquire a fund of information from the particular study of patterns of development of a number of model virus systems using both DNA and RNA

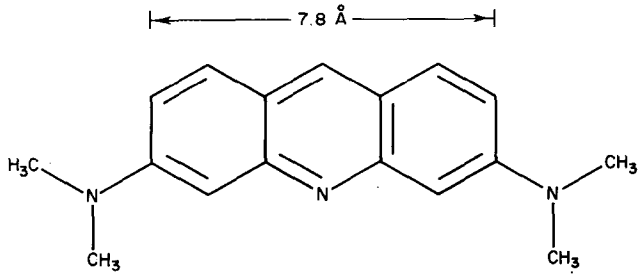


FIG. 1. Acridine orange molecule.

viral agents, which will have more general applications. In addition, some cautious extrapolation to newly isolated viral agents may be in order.

II. Acridine Orange as a Specific Stain

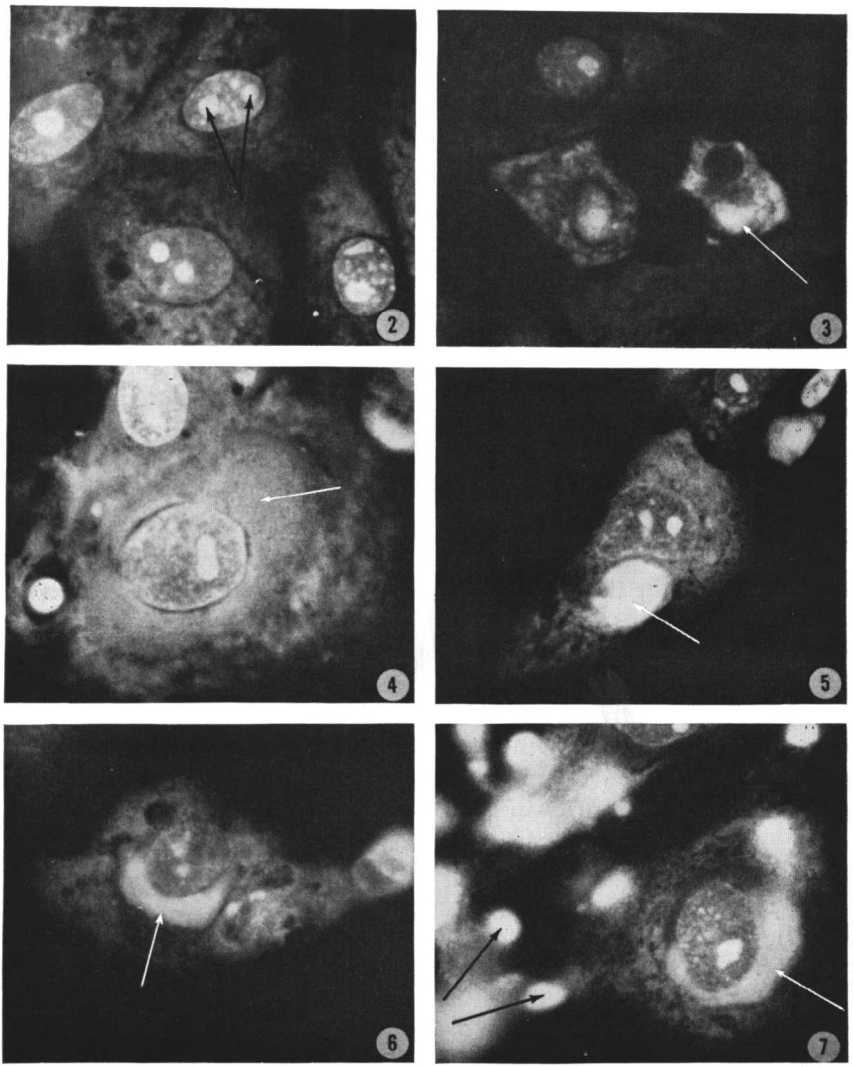
A. Acridine Orange as a Cytochemical Stain for Intracellular Viral Nucleic Acids

In recent years two very versatile techniques have added much to our knowledge of the intracellular localization of viruses and their cycles of development: one is the fluorescent antibody technique of Coons and Kaplan (1950), a highly specific method for locating viral antigens, but outside the scope of this review. The reader is referred to Coon's article in the *International Review of Cytology* (1956). The other is a sensitive cytochemical procedure for identifying nucleic acids, again utilizing fluorescence microscopy, which is based on the use of the fluorochrome acridine orange as a specific stain. This technique owes much of its present-day popularity among virologists and cytologists to the developments of Armstrong and Niven (1957) and von Bertalanffy and Bickis (1956).

In brief, when suitably fixed tissues or virus preparations are stained

with the fluorochrome under carefully controlled experimental conditions and viewed by blue-violet light, the components containing deoxyribonucleic acid (DNA) fluoresce a brilliant yellow-green while those containing ribonucleic acid (RNA) exhibit a flame-red fluorescence. Although most fixatives other than those containing osmium tetroxide or picric acid will allow adequate staining, the most brilliant fluorescence is usually obtained after brief fixation in Carnoy's fluid (60% ethanol, 30% chloroform, 10% glacial acetic acid). We have found that a pH of 4.0 and a fluorochrome concentration of 0.01% in McIlvaine's citric acid disodium phosphate buffer are most satisfactory for yielding maximum brilliance with most sharply contrasted differential fluorescence of nucleic acid components. Confidence in the identification of nucleic acids depends ultimately on the satisfactory performance of relevant enzyme digestion tests: e.g., the development of the yellow-green stain characteristic of DNA is prevented by prior treatment of fixed preparations with DNase (deoxyribonuclease) but not by RNase (ribonuclease), while the flame-red of RNA is prevented by RNase but not by DNase.

The normal cellular pattern of nucleic acid distribution finds DNA occurring solely in the chromosomes in the nucleus. RNA can also be found in the nucleus, most abundantly in the nucleoli, but the greatest source of cellular RNA is in the cytoplasmic ribosomes. In addition, two other varieties of RNA of lower molecular weight can be distinguished in cells, soluble or transfer RNA and messenger or informational RNA. All the known viruses, whether their host is an animal, plant, insect, or bacterium, have been found to contain only one type of nucleic acid, either DNA or RNA. However, the sites within the cell where viral synthesis and maturation can occur vary from one group of viruses to another. Although most DNA viruses mature in the nucleus of infected cells while RNA-containing ones are more at home in the cytoplasm, this is no hard and fast rule, and we have, for example, among the DNA viruses the pox viruses which appear to form entirely within the cytoplasm (Gaylord and Melnick, 1953; Morgan *et al.*, 1954), the simian papova virus, vacuolating SV40 (Melnick, 1962), which has well-developed nuclear and cytoplasmic phases (Hsiung and Gaylord, 1961; Gaylord and Hsiung, 1961; Mayor *et al.*, 1962a), and the adenoviruses which are completely formed within the nucleus (Morgan *et al.*, 1956a; Harford *et al.*, 1956). Among RNA viruses the replication of reo-virus appears to proceed in the cytoplasm without any visible nuclear changes (Rhim *et al.*, 1962) while the ECHO viruses show definite cytochemical evidence of nuclear involvement (Jamison *et al.*, 1962) and influenza virus is completed only at the plasma membrane (Morgan *et al.*, 1956b). The cytochemical patterns of the virus-host cell interaction obtained



FIGS. 2–7. Normal monkey kidney cells stained by the acridine orange technique (black and white copies of color originals). Fig. 2. Reddish tinge in the nucleoli (black arrows) indicates presence of RNA; flame-red color, cytoplasmic RNA; and yellow color, nuclear RNA. Fig. 3. Monkey kidney cells 16 hours after infection with reo-virus, type 1 (strain 716). Note the increase of RNA in the cytoplasm (white arrow). Fig. 4. Cells 24 hours after the infection. Note green-staining protein material in the cytoplasm (white arrow) and increased cytoplasmic RNA. No pyknosis and no nuclear change. Fig. 5. Cells 48 hours after infection. Brilliant cytoplasmic RNA staining (white arrow) is present in 50% of the cells. Fig. 6. Cells 54 hours

after acridine orange staining are more clearly delineated when grouped according to type of viral nucleic acid and the established sites of replication and maturation.

1. *RNA Viruses*

a. Viruses Demonstrating a Cytoplasmic Phase Alone. Anderson *et al.* (1959) observed the growth of West Nile encephalitis, an arbor virus which contains RNA, in monolayer cultures of infected chicken fibroblasts for a period of 6 days. The major sites of activity were cytoplasmic and this activity appeared in the form of increased granularity and staining brilliance of the RNA. Apart from increased prominence and brilliance of the nucleolus, the general nuclear morphology remained virtually unchanged until cell death.

Reo-virus, formerly known as ECHO 10, a somewhat larger RNA virus which causes respiratory and enteric diseases in man and animals, has been extensively studied with acridine orange staining (Rhim *et al.*, 1962; Gomatos *et al.*, 1962) and is an excellent example of a replication cycle which appears to take place without any morphological evidence of nuclear involvement.

With the acridine orange staining technique, changes in the amount and distribution of cytoplasmic RNA could be detected as early as 16 hours after infection. Figures 2–7 are black and white copies taken from the Kodachrome originals of sequential studies in our laboratory. The appearance of normal monkey kidney cells is shown in Fig. 2. The nucleus exhibited an even green fluorescence lightly flecked with yellow, indicative of chromosomal DNA, the nucleoli a reddish stain, demonstrative of RNA. The cytoplasm was uniformly flame-red. Figure 3 shows monkey kidney cells 16 hours after infection with reo-virus; increased cytoplasmic RNA was readily visible. Twenty-four hours after infection a pale green cytoplasmic inclusion surrounding the nucleus and often extending throughout the cytoplasm in long threads could be observed (Fig. 4). Bright points of RNA staining were visible within this green inclusion. Most nuclei gave normal staining reactions at this stage. Forty-eight hours after infection, a brilliant red cytoplasmic inclusion had developed in approximately 50% of the cells (Fig. 5). It appeared

after infection. Note a marked increase of brilliant cytoplasmic RNA, concentrated into a definite area (white arrow). Release of RNA material is evident at the cell borders. Fig. 7. Cells 72 hours after infection. Note inclusions in cytoplasm, surrounding nucleus (white arrow). Brilliant cytoplasmic RNA is demonstrated within these inclusions in 80 % of the cells. The nuclei appear normal. There is no enhancement of RNA staining in the nucleoli. Many rounded and shrunken cells with pyknotic nuclei are seen (black arrows).

to develop from within the green inclusion which was still evident in many cells. By 54 hours the cytoplasmic inclusion was large and brilliantly stained for RNA. Release of RNA-containing material was also readily visible at the plasma membranes (Fig. 6). By 72 hours 80% of the cells were involved, and in addition to the previous changes, cell shrinkage, and nuclear pyknosis were evident (Fig. 7).

At no stage in the cycle was enhanced RNA staining of the nucleolus observed. In fixed preparations demonstration of the green cytoplasmic inclusion was completely prevented by prior treatment with pepsin

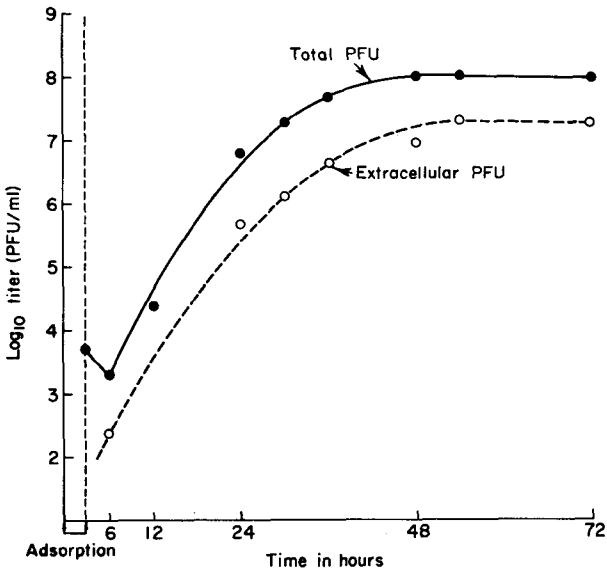


FIG. 8. Growth cycle of reo-virus type 1 in monkey kidney tissue culture.

alone but not by DNase or RNase, a result indicating its protein nature. The red inclusions were prevented by RNase alone, but not by DNase or pepsin, and yellow staining of nuclear material (chromatin) was prevented by DNase alone.

The reo-virus growth cycle was determined in monolayers of monkey kidney cells by plaque assay (Fig. 8). It was shown that the eclipse phase for reo-virus was approximately 6 hours, that the increase of infectious virus was exponential and completion of replication required a minimum of 54 hours, when 40–50% of the cells showed cytopathic effects readily visible in the light microscope. Only 20% of the total virus was found to be extracellular throughout most of the growth cycle. This appearance of virus correlated well with the demonstration by

acridine orange staining of the increase and persistence of additional RNA in the cytoplasm. The prolonged growth cycle and tendency to accumulate within the host cell are quite striking properties of reo-virus. The apparent lack of nuclear participation in the accumulation of specific viral protein and nucleic acid is in contrast to the behavior of another RNA virus, poliovirus, where definite nucleolar abnormalities have been reported (Stuart and Fogh, 1961; Mayor, 1961a). This suggests that the cytoplasm alone may be capable of initiating and sustaining the production of reo-virus.

b. Viruses Demonstrating Both Nuclear and Cytoplasmic Phases. The majority of the small (20–30 mμ diameter) RNA viruses recently grouped

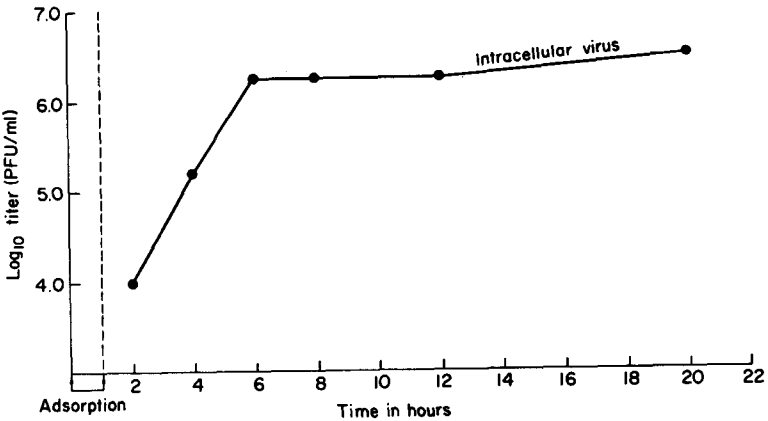


FIG. 9. Growth cycle of poliovirus type 1 attenuated strain in monkey kidney tissue culture.

as the PICORNA viruses (International Enterovirus Study Group, 1963) would appear to fit into this category. These viruses, which include the polio, ECHO, Coxsackie, and rhinoviruses, consist solely of an RNA core enclosed within a protein capsid exhibiting cubic symmetry. Poliovirus is a typical example where cytochemical procedures reveal abundant evidence of both nuclear and cytoplasmic involvement (Mayor, 1961a).

Using monkey kidney cells infected with poliovirus, cytopathic changes were always well established within 6 hours of inoculation. Using the acridine orange stain, changes could be detected in the distribution of nuclear DNA as early as 3 hours after inoculation. The appearance of normal kidney cells did not differ from those examined in the reo-virus experiments (Section II, A, 1, a and Fig. 2). By 3 hours after infection there was a marked clearing in the nuclear background together with aggregation of the DNA which appeared to stain very

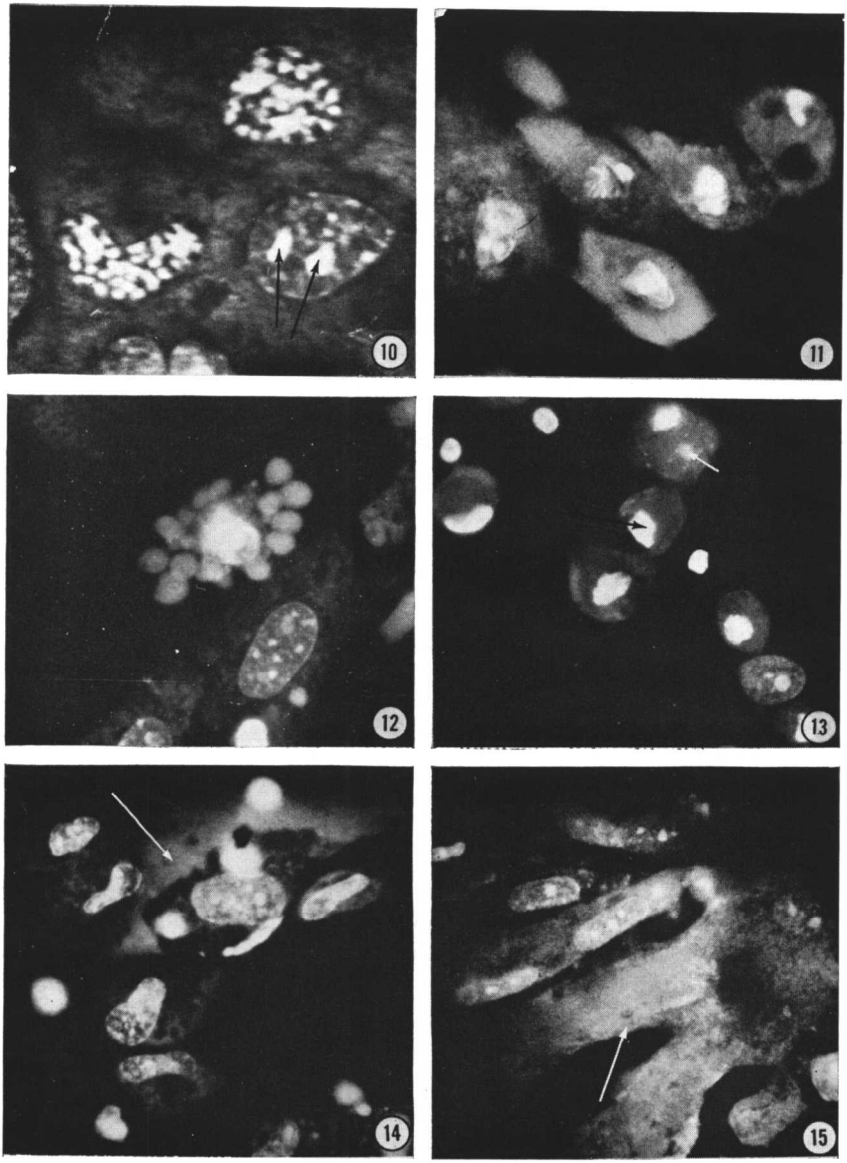


FIG. 10. Monkey kidney cells 3 hours after inoculation with poliovirus type 2 (black and white copy of color original). Acridine orange technique. Nuclear clearing and clumping of DNA are evident. Nucleoli (black arrows) are stained bright red.

FIG. 11. Monkey kidney cells 6 hours after inoculation with poliovirus type 2 (black and white copy of color original). Acridine orange technique. Brilliant cyto-

brightly (Fig. 10). The nucleoli were stained a more brilliant red than in the control cultures indicating increased nucleolar RNA activity. There was no detectable increase in the intensity of staining of cytoplasmic RNA. However, by 3 hours after inoculation a definite increase in cytoplasmic RNA above the level usually encountered in normal monkey kidney cells (except those undergoing mitosis, which stain brilliantly) could be detected in infected cells. Many cells were beginning to elongate at this stage and nuclear clearing with DNA clumping were very marked.

Six hours after inoculation when intracellular virus had reached a maximum (Fig. 9) a maximum brilliance in cytoplasmic RNA staining was encountered with acridine orange, usually involving 50% of the cells (Fig. 11). Many cells were beginning to round up, the nuclei becoming eccentric and pyknotic with brilliant and concentrated DNA staining. By 8 hours, about 80% of the cells were rounded, and marked release of brilliant RNA material was observed in blebs at the plasma membrane (Fig. 12). This was interpreted as indicating release of poliovirus, which is known to reach its maximum at 8 hours in monkey kidney monolayers (Howes and Melnick, 1957). (The RNA in these fixed preparations was susceptible to digestion with RNase without the necessity of proteolytic enzyme treatment at all stages of the growth cycle.)

By this time many of the rounded cells were completely void of RNA and were conspicuous for their shrunken appearance and for

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plasmic RNA staining is present in 50% of the cells. Nuclei stain brightly for DNA and are pyknotic.

FIG. 12. Monkey kidney cells 8 hours after inoculation with poliovirus type 1 (black and white copy of color original). Acridine orange technique. Release of RNA material at the plasma membrane in blebs is evident.

FIG. 13. Shrunken monkey kidney cells 8 hours after inoculation with poliovirus type 1 (black and white copy of color original). Acridine orange technique. Most of the virus has been released from these cells. Note distribution of nuclear DNA material (black arrow) and bright RNA chunks in the cytoplasm of the cell at the top of the field (white arrow).

FIG. 14. Human embryonic fibroblasts 12 hours after infection with vesicular stomatitis virus (black and white copy of color original). Acridine orange technique. The cytoplasm of infected cells stains brilliantly for RNA. RNA material has accumulated at the cell periphery (white arrow). Nuclear involvement and clumping of DNA is evident.

FIG. 15. Human embryonic fibroblasts 16 hours after infection with vesicular stomatitis virus (black and white copy of color original). Acridine orange technique. Syncytial formation is evident (white arrow). Syncytia are brilliantly stained for RNA.