HEATHER DONALD MAYOR*
JOSEPH L. MELNICK**

Department of Virology and Epidemiology, Baylor University College of Medicine Houston, Texas

INTRACELLULAR AND EXTRACELLULAR REACTIONS OF VIRUSES WITH VITAL DYES†

Vital dyes, long used in cytologic studies of living cells, are now assuming increasingly important and diverse roles in virus research. They are employed as visual indicators of infection in virus assays, and their involvement in the photo-inactiviation of viruses is well established. In particular, studies with such dyes as neutral red, toluidine blue, proflavin, and acridine orange have been most fruitful. The fluorochrome, acridine orange, is of special importance because it has the dual capacity to act as a vital stain and as a cytochemical reagent for the detection of nucleic acids. Moreover, under conditions of controlled pH and concentration, acridine orange can differentiate, at the microscopic level, between RNA, double-stranded DNA, and single-stranded DNA.

In this paper, we have brought together several aspects of our recent work related to the use of vital dyes in virus research.[1,2] Included are studies on: the photo-inactivation enhancing effect of dyes incorporated into developing poliovirus; the multiplication of poliovirus using correlated cytochemical and fluorescent antibody techniques; and the use of acridine orange as a differential reagent for nucleic acids in purified viruses.

THE INCORPORATION OF NEUTRAL RED AND ACRIDINE ORANGE INTO DEVELOPING POLIOVIRUS AND THE PHOTOSENSITIVITY OF VIRUS PRODUCED BY STAINED CELLS

It has been observed in several laboratories that virus plaques may be reduced in size and number in the presence of the vital dye, neutral red. Because inhibition apparently occurs somewhat irregularly, experiments were designed to determine what added influence light might have on the growth of poliovirus in cells containing neutral red. The combined action of light and acridine orange was also tested in a similar fashion.

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*Professor of Virology and Epidemiology. Formerly, Fellow (National Research Council), and subsequently Professor of Epidemiology, Department of Preventive Medicine, Yale University School of Medicine 1941-1957.

**Assistant Professor of Virology.

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MATERIALS AND METHODS

Tube cultures of monkey kidney cells were grown in Hanks' medium plus 2% calf serum and 0.5% lactalbumin hydrolysate, until cell sheets, representing about 10^6 cells, were formed. Melnick-Earle's medium, was used for maintenance. Virulent (Mahoney) and attenuated (LSc) strains of poliovirus were compared in the test system. Each tube was inoculated with 1 ml. containing $10^{5.5}$ PFU of virus. After adsorption for one hour, the cultures were washed with warm medium and 1 ml. of maintenance medium plus dye was added.

Dyes. Either neutral red or acridine orange was incorporated into the maintenance medium at concentration levels ranging from $10^{-5.1}$ to $10^{-5.4}$ About two hours were allowed for dye to be taken in by the cells before any light was applied.

Light exposure. An effort was made to prevent undue exposure to light of the developing (or harvested) virus. Necessary manipulations were carried out in subdued lighting and culture racks were enveloped in aluminum foil to prevent extraneous light from reaching the cells. Cultures were kept in continuous contact with dye, in the dark, for a period of eight hours, insuring that virus development would take place in stained cells. Some of the tubes were carried in darkness for the entire eight hours; others, taken at different periods, were exposed to light for one hour at 37°C. For this purpose, three 20 watt "daylight" type fluorescent lighting tubes were placed eight to nine inches away from the exposed cultures. Following this, the cultures were returned to the dark for the remainder of the eight-hour test period. At the end of eight hours, all tubes were harvested and tested for infectious virus. For control, "normal" mature virus (i.e. produced in the absence of any dye) was incubated with dye in the dark and then exposed to light for one hour before testing for infectivity.

Harvest and assay. At harvest, each tube was subjected to two cycles of freezing and thawing. After the second thawing the harvests from five tubes collected at each time interval were combined, centrifuged to remove cell debris, and the supernates titrated by the plaque technique. Details of the procedure have been described.[8]

RESULTS

In the presence of either neutral red or acridine orange, poliovirus production was inhibited to some extent, even in the dark. With the addition of light, however, inhibition was markedly increased (Table 1). This effect was easily demonstrable with either dye, neutral red or acridine orange, and no differences were noted in tests with virulent and attenuated Type 1 strains of virus. The time at which light was applied was not a factor in the inactivating process and it was found that light enhancement occurred after harvest as well as during the virus developmental period, provided that the harvested virus had been grown in the presence of dye. Light had no effect on virus produced in the absence of dye even when the latter was added before exposure to light.

DISCUSSION

The reduction in infective virus was due to the effects of light on virus developed in the presence of neutral red, since the dye and light had no effect on the mature virus grown in the absence of neutral red. Exposure to light resulted in a marked reduction in infectivity of virus which had been developed in the dark in cells containing neutral red or acridine orange. The possibility that the effect was related to alterations produced in the cells seems remote, in view of other evidence. Thus, it appears most likely that neutral red becomes incorporated into poliovirus developing in the presence of the dye.

TABLE 1. EFFECT OF LIGHT ON VIRUS GROWN IN PRESENCE OF NEUTRAL RED IN THE DARK

Neutral red concen.	*PFU titers after incubation in dark 1 hour*		*PFU titers after incubation in light 1 hour*		
	Av. 3 exp.	*Log_{10} inhibition in dark*	*Av. 3 exp.*	*Log_{10} inhibition in light*	*Log_{10} inhibition light-dark**
Mahoney:					
0	5.75	0	5.73	0	0
1:240,000	4.4	1.35	<1	>4.73	>3.38
LSc:					
0	5.1	0	5.1	0	0
1:120,000	3.7	1.4	1.75	3.35	1.95

* Inhibition due to light over and above that due to neutral red alone plus one hour incubation at 37° C. in the dark.

Our experiments, when compared with previous work of other investigators, indicates a similarity between the biological effects of neutral red and of other photodynamically active dyes such as proflavin, toluidine blue, and acridine orange. This is not surprising when one considers the formulae of these compounds (Figure 1). Proflavin is an acridine and has the formula shown with a carbon in the X-position. Neutral red, a quinonimide dye, has a nitrogen in the X-position. Toluidine blue is yet another example of a quinonimide dye. The similarities between the formulae are obvious. Tricyclic photodynamically active dyes have the general formula shown at the bottom of Figure 1, "X" indicating carbon or nitrogen and "Y" indicating sulphur, oxygen, or nitrogen. All are basic heterotricyclic compounds with an affinity for nucleic acid.

Hiatt and his coworkers[4,5] have found that enteroviruses cannot be inactivated by toluidine blue and white light, whereas vaccinia and adenoviruses are readily inactivated under the same conditions. The susceptibility of phage to the action of an active dye and light appears from their work to be linked to the permeability of the virus, since there is a correlation between these experiments and osmotic permeability of the phage as measured by osmotic disruption experiments.

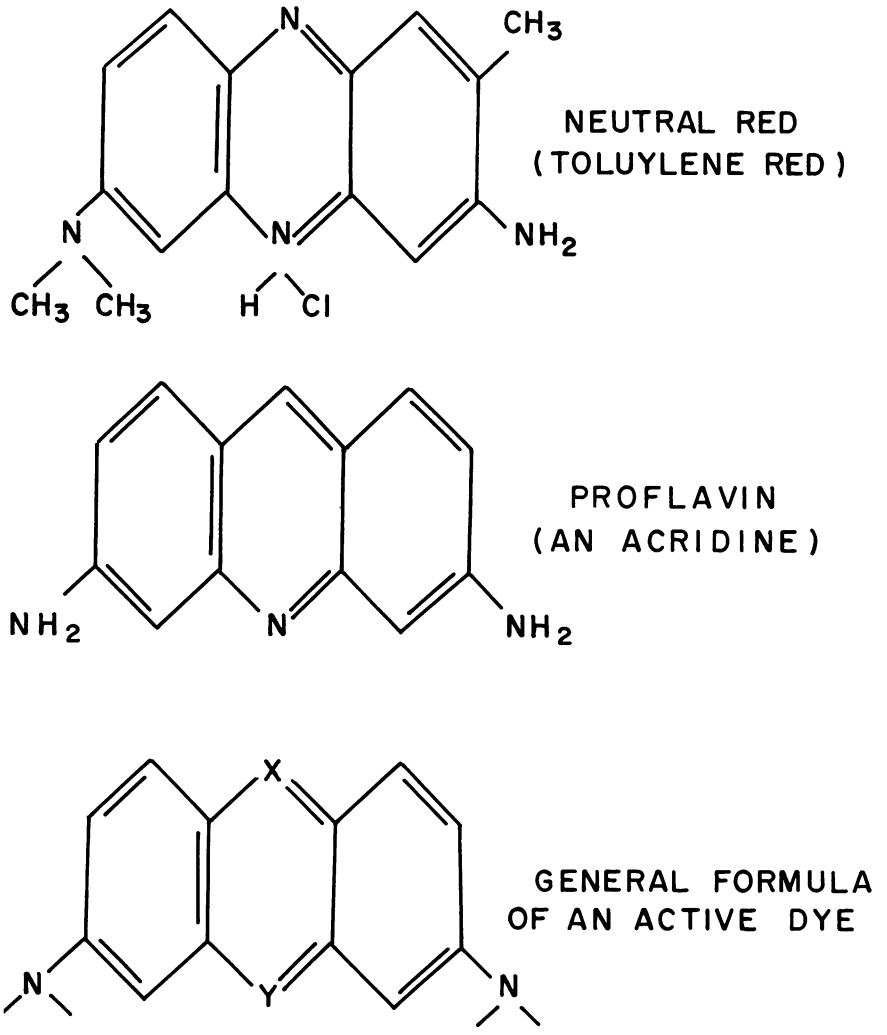

FIG. 1. Structural formulae of photodynamically active dyes.

Schaffer[6] has recently found that proflavin cannot attach to mature poliovirus particles, whereas it can be incorporated into developing poliovirus. In the course of our study, a similar sort of phenomenon was found to occur with neutral red: poliovirus is not inactivated by neutral red and light in the absence of cells, but virus grown in cells containing neutral red may be inactivated by light, indicating that an incorporation of the dye into developing poliovirus may occur. We have found that acridine orange yields similar results, and as described below, have obtained direct microscopic evidence of the incorporation of the dye into the virus.

Experiments performed with proflavin and other acridines as viral inhibitors have been carried out, until recently, without reference to the part that light may play in this inhibition. The possibility of incorporation of the dyes into the virus particle and its subsequent inactivation by light was not considered by previous workers. Thus Ledinko[7] demonstrated the cytopathogenicity of poliovirus to be unaffected by proflavin, and we have observed that the same is true using neutral red, unless near-toxic levels of dye are used. Both proflavin and neutral red are capable of inhibiting the production of infective poliovirus even in subdued lighting. It is unknown as yet exactly what part the dye alone and what part added light play in the production of non-infective virus. In the experiments of Ledinko and Schaffer, virus produced in the presence of proflavin, and shown by Schaffer to contain the dye, was largely non-infective, but the effect of laboratory lighting was not excluded. In later experiments carried out in the dark, Schaffer has also found that proflavin produces only slight inhibition in the production of infective poliovirus (personal communication).

CYTOCHEMICAL AND FLUORESCENT ANTIBODY STUDIES ON THE GROWTH OF VIRUSES

Virulent and attenuated polioviruses. In the past few years two versatile techniques have added much to our knowledge of the intracellular localization of viruses and of their cycles of development: one is the fluorescent antibody method of Coons and Kaplan[8] which locates specific viral antigens, the other is the acridine orange technique developed independently by Armstrong[9] and by von Bertalanffy and Bickis,[10] a technique which can be used as a cytochemical method for demonstrating intracellular nucleic acids. A number of investigators have used one or the other of these techniques in studies of host-virus relationships. For fuller citations of the literature, see other references.[11,12]

Recently, interest has been focused on the effect of vital, photodynamically active dyes (of which acridine orange is an example) on the infectivity

of virus particles. The action on both mature and developing particles has been studied. LoGrippo and Basinski[18] have shown that the whole poliomyelitis particle, in contrast to the T3 bacteriophage, is not inactivated by the action of acridine orange and ultraviolet light. Hiatt[14] has demonstrated that the enteroviruses are unaffected by exposure to toluidine blue and light, while herpes B, vaccinia, rabies, simian vacuolating viruses, and, to a lesser extent, adenoviruses and reovirus, *are* inactivated. As noted above and in a previous report,[1] mature poliovirus incubated with neutral red and exposed to white light showed no reduction in titer, but virus *grown* in cells containing the dye was inactivated by exposure to light, suggesting incorporation of the dye into the virus particle. Schaffer[6] obtained direct evidence for the incorporation of proflavin into poliovirus during its growth in tissue culture and assumed that the dye becomes bound to the RNA of the virus particle. Our experiments described below are an attempt to present an integrated cytochemical and biological study on the developing poliovirus particle in tissue culture.

MATERIALS AND METHODS

The sequence of changes in coverslip tissue culture monolayers of monkey kidney cells infected with poliovirus (virulent and attenuated strains) has been followed by cytochemical means (acridine orange staining) and by the fluorescent antibody technique. Changes have been followed closely during a single cycle of infection and beyond, both at 37°C and at 30°C. In Carnoy-fixed preparations, stained with 0.01% acridine orange at pH 4.0, material identified as DNA stained a brilliant greenish-yellow and was susceptible to DNAase; material identified as RNA stained a brilliant flame red and was susceptible to RNAase. Techniques in current use in our laboratory for controlled nuclease digestion tests have been described together with our method for growing poliovirus in the presence of acridine orange.[15] The direct fluorescent antibody procedure[10] using immune monkey globulin conjugated with fluorescein isothiocyanate was employed. Reichert fluorescence equipment and suitable filters were used.

RESULTS

In general, no significant differences between the virulent and attenuated strains of poliovirus could be detected with acridine orange or by the fluorescent antibody technique. When incubated at 37°C. virus protein (antigen) and increased cytoplasmic RNA were detectable three hours after infection and both reached a maximum in staining intensity about six to eight hours after inoculation. At no time was specific antigen detected in the nucleus of infected cells, although marked nuclear alterations and enhanced nucleolar staining were noted by the acridine orange technique three hours after inoculation. At eight hours, evidence of virus

release was observed with both techniques. Figures 2, 3, 5 and 7 show some of our observations with these techniques. Parallel titrations of intracellular virus (Fig. 8) revealed a gradual increase in the amount of virus which reached a maximum at six to eight hours, in excellent agreement with the cytochemical and fluorescent antibody findings.

Similar results were obtained at 30°C., but intracellular changes occurred approximately four hours later than those observed at 37°C. The peak of staining intensity occurred approximately twelve hours after inoculation. In contrast to growth at 37°C., very little virus release was observed at 30°C. (Fig. 6) at any time. However, release could be obtained from 30°C. cultures by placing them at 37°C. for an additional hour at the end of the incubation period. Actually, a brilliant picture of virus release was then observed (Fig. 4). Figure 8 shows the slower growth rate at 30°C., in agreement with the cytochemical and fluorescent antibody findings. This figure also shows titers of intracellular virus which were reached in the presence of acridine orange. The small amount of inhibition found was probably due to photodynamic inactivation by stray light.

DISCUSSION

Fluorescent antibody and acridine orange techniques are both highly sensitive means for following the growth of poliovirus in tissue culture. The limit of resolution of the fluorescent antibody technique is in the vicinity of 5×10^{-15} gm. antigen,[16] which corresponds to some hundreds of poliovirus particles. Mayor and Diwan[15] have estimated the sensitivity of the acridine orange technique for small RNA viruses to be of the order of 50 particles. However, the specificity of the acridine orange method —particularly for the study of RNA viruses against a background of cellular RNA—is dependent on changes in density and distribution of the nucleic acid and is not as highly specific as the fluorescent antibody technique.

Anderson *et al.*[17] have pointed out that in the case of DNA viruses a relative insusceptibility to DNAase digestion is a constant feature of animal virus infections and that resistance to the nuclease could be abolished by pretreatment with a proteolytic enzyme. Here we have a useful tool for distinguishing between the host cell DNA and the viral DNA. However, so far no cytochemical method has been found for distinguishing between viral and cellular RNA. Influenza,[17] Rous sarcoma,[18] and poliovirus,[19] are RNA viruses which in the fixed state have proved susceptible to RNAase. Perhaps a method of fixation which will allow digestion of cellular RNA while leaving viral RNA intact will be developed.

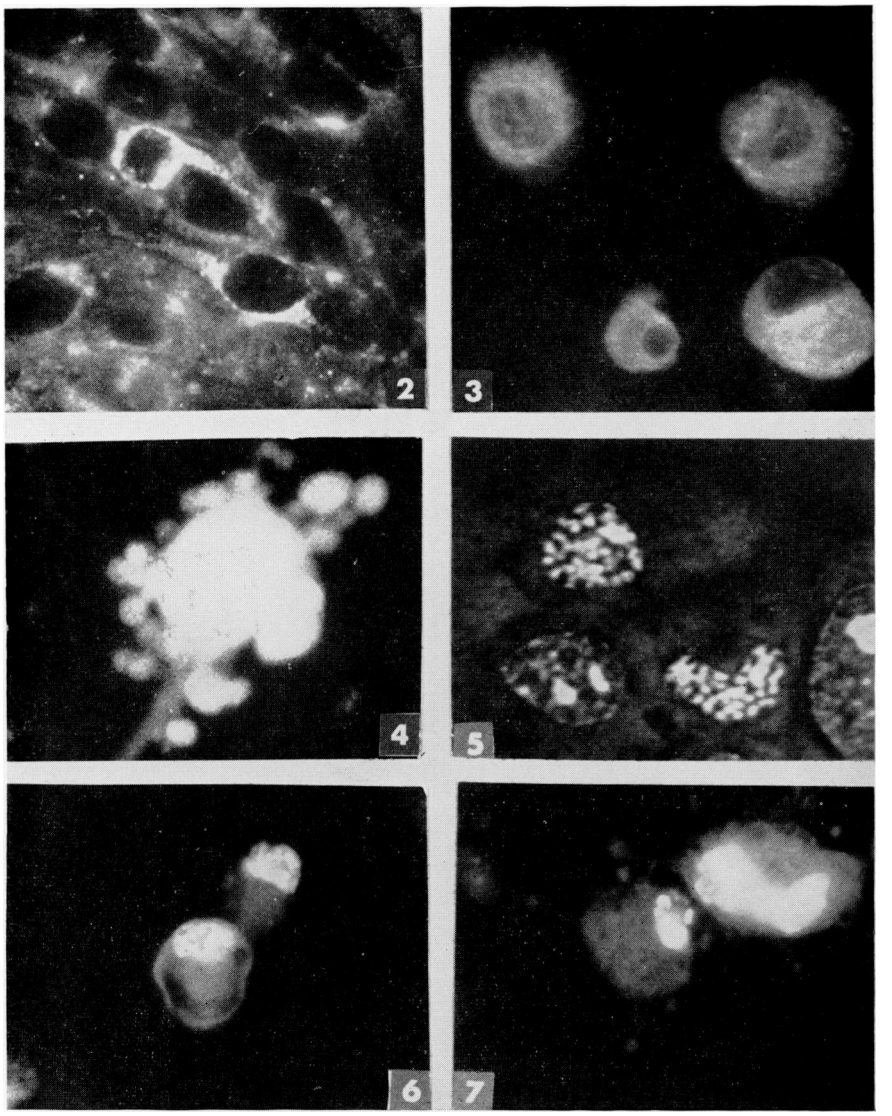

FIG. 2. Monkey kidney cells 3 hours after infection with LSc type 1 attenuated poliovirus stained by the direct fluorescent antibody technique for viral antigen. Note bright fluorescent staining in a ring around the nucleus in 2 cells at the center of the field. X1,000

FIG. 3. Monkey kidney cells 8 hours after infection with LSc type 1 attenuated poliovirus, incubated at 30° C. Note swelling of cells, integrity of nucleus and diffuse antigen. X1,000

FIG. 4. Monkey kidney cells 21 hours after infection with MEF_1 type 2 virulent poliovirus, incubated 20 hours at 30° C. and then placed for 1 hour at 37° C. Note intense fluorescence completely filling the cell and extensive release of antigen. X1,200

FIG. 5. Monkey kidney cells 3 hours after inoculation with Y-SK type 2 attenuated virus. Nuclear clearing and clumping of DNA are evident. Nucleoli are stained bright red. X2,000

FIG. 6. Monkey kidney cells incubated at 30° C. for 8 hours after inoculation with Mahoney type 1 virulent poliovirus. Note three dimensional appearance of cells and peripheral RNA staining; also absence of virus release. X900

FIG. 7. Monkey kidney cells 8 hours after inoculation with LSc type 1 attenuated virus. Note rounded cells full of RNA and eccentric brilliantly stained nuclei. Release of RNA material is evident at the cell walls. X2,000

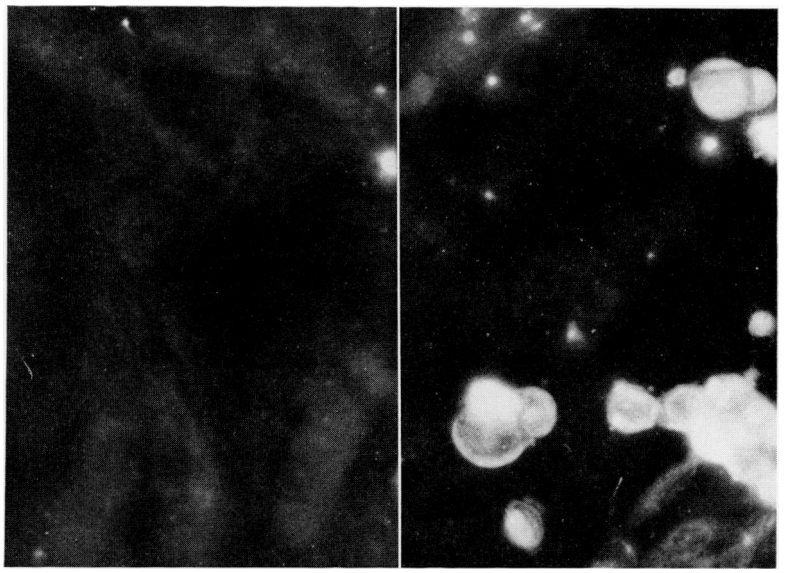

FIG. 9. Eight-hour yields of type 2 poliovirus stained with fluorescent antibody. Photomicrograph left: virus culture in presence of guanidine. Photomicrograph right: virus-infected cells in control culture.

Inhibitory action of guanidine on poliovirus multiplication. Guanidine, $(NH_2)_2CHN$, is an effective inhibitor of poliovirus multiplication in monkey kidney cell cultures.[20] As little as 60 μg per ml. of guanidine-HCl will reduce the yield of virus by five logs. Extensive studies on the mode of action of guanidine have been carried out in our laboratory in an attempt to elucidate the site at which the compound acts in the virus multiplication cycle.[21] The inhibitor prevents cytopathic changes in infected cultures and interferes with the production of infective polio-

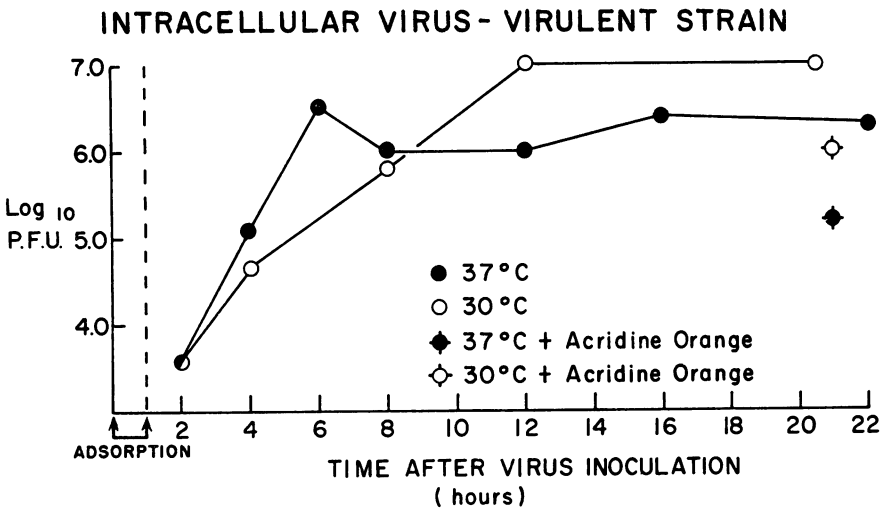

FIG. 8. Growth of poliovirus type 2, MEF_1 virulent strain, at 37° C. and at 30° C. Titers reached in presence of acridine orange after 21 hours at 37° C. and 30° C. are included.

virus. Staining with acridine orange and with fluorescent antibody proved useful in determining that guanidine can inhibit both the production of cytoplasmic RNA associated with poliovirus infection, and the production of antigenic viral protein.

The following experiment was carried out to elucidate further the nature of guanidine inhibition of poliovirus multiplication. Monkey kidney cells were grown on coverslips in Leighton tubes. Three sets of such cultures were used in each test: into one set, virus alone was inoculated; into another, virus plus 100 μg of guanidine; a third set, without virus and without guanidine, served as controls. The virus inocula consisted of 0.1 ml. of undiluted poliovirus Type 2 MEF_1 (titer $10^{8.1}$ PFU/ml.). One hour was allowed for adsorption after which excess virus was washed off.

After five hours, tubes were harvested for titration and several were stained with acridine orange. After eight hours, additional tubes were harvested and several were stained with fluorescent antibody, using the direct method.

The results obtained are shown in Table 2. When stained for antigen by the fluorescent antibody technique, controls were uniformly negative. Cultures of virus grown in the presence of guanidine in monkey kidney cells were also negative. However, virus grown for eight hours showed bright fluorescence (Fig. 9), but prior treatment of the cells with specific antiserum inhibited the staining. These experiments indicate that the antigenic protein coat of the virus was not being formed under guanidine inhibition of infective virus.

TABLE 2. GUANIDINE INHIBITION OF TYPE 2 POLIOVIRUS

	Control		*100 μgm./ml. guanidine*	
Time of harvesting	5 hrs.	8 hrs.	5 hrs.	8 hrs.
Log PFU titer	6.7	7.2	3.8	4.1
Log inhibition	—	—	2.9	3.1

In cell controls similar to those in the fluorescent antibody staining experiment, acridine orange staining showed a uniform but not intense red staining of the cytoplasm. Cells containing virus grown under normal conditions showed an intense red staining of the cytoplasm suggesting an active production of RNA over that of controls, as described above. Cells containing the small amount of virus which developed in the presence of 100 μg of guanidine for five hours showed the same pale red staining as the controls, suggesting that no marked change in cytoplasmic RNA had taken place under the conditions. It follows from these experiments that guanidine inhibits both the production of the protein antigen of the virus and the increase of red-staining cytoplasmic RNA associated with viral synthesis.

Guanidine is a remarkable inhibitor of poliovirus multiplication in that structurally it is one of the simplest molecules capable of inhibiting the development of a virus. Substitution of any of the nitrogen groups yields an inactive molecule, indicating the specific nature of its action.[21]

ACRIDINE ORANGE STAINING OF PURIFIED VIRUSES

Poliovirus and tobacco mosaic virus (RNA). The ability to see, measure, and count the actual elementary bodies of viruses is funda-

mental from a biophysical standpoint and necessary for their identification or for quantitative correlation with their biological effects. Although some of the large viruses, such as vaccinia, can be identified under the light microscope with suitable staining, and the filamentous forms of influenza virus are readily visible by dark field illumination, it is generally necessary to employ electron microscopy to determine shape and to measure directly the size of most virus particles.

Using the acridine orange staining technique, Anderson, *et al.*[17] were able to identify individual heads of T_2 bacteriophage particles with the fluorescence microscope. As the DNA content of T_2 bacteriophage is about 2×10^{-10} μgm per particle,[22] this finding represents a high degree of sensitivity for the detection of DNA by cytochemical methods. Anderson, *et al.* were unable to detect individual RNA virus particles and considered their identification beyond the limit of sensitivity of the technique. Epstein and Holt[18] examined purified preparations of Rous sarcoma virus in the fluorescence microscope and established RNA as the sole nucleic acid present. Correlated studies in the electron microscope localized the RNA in the nucleoid of the virus particle. It is obvious that the use of the fluorochrome, acridine orange, as a vital dye presents an intriguing possibility for visualizing individual virus particles by virtue of their vitally stained nucleic acids. We have carried out experiments on these lines with purified poliovirus and tobacco mosaic virus.

MATERIALS AND METHODS

Purification and concentration of virus. A single procedure was followed for both poliovirus and AO-poliovirus (grown in the presence of acridine orange) except that in the latter case maximum precautions were observed at every stage of the process to protect the infective fluids from inactivation by light. The virus pools were clarified by low speed centrifugation and concentrated by overnight treatment at 4°C with polyethylene glycol (Carbowax 20 M). Aliquots of the virus suspensions, each volume approximately 70 cc., were then purified and further concentrated chromatographically by adsorption to and elution from a calcium phosphate column. Final concentration was achieved by ultracentrifugation.

Studies of tobacco mosaic virus (TMV) were carried out on a sample of purified concentrate prepared and sent to us in 1943 by Dr. Wendell M. Stanley.

Staining technique. Small droplets of virus concentrates were placed at the center of 11 x 22 mm. coverslips and allowed to dry thoroughly in air. When desired, fixation was carried out on these previously dried preparations. Fixation with chilled absolute methanol for 15 minutes was found most satisfactory. In an additional experiment the following concentrations of methanol were used: 95%, 70%, 50%, 40%, 30%. Carnoy's fluid was also employed as a fixative in a number of cases. The standard procedures for acridine orange staining and for nuclease digestion used in this laboratory[11,15] were employed. The staining was with 0.01% dye at pH 3.8-4.

RESULTS

The methods followed for purification and concentration of virus were found to be entirely satisfactory. Electron microscopy showed that the poliovirus concentrates consisted entirely of spherical particles approximately 30 mμ in diameter while the TMV concentrates yielded typical rods. Table 3 gives the titers of virus (in PFU per ml.) obtained for poliovirus and AO-poliovirus before and after the purification and concentration process. In each experiment the final volume, after ultracentrifugation, was approximately 1/100 the volume of the original tissue culture fluid. Table 3 shows clearly that in the case of poliovirus the log increase in titer of

TABLE 3. TYPICAL TITERS OF POLIOVIRUS AND AO-POLIOVIRUS FRACTIONS

Material	*Poliovirus* $\log_{10}$ *PFU/ml.*	*AO-Poliovirus* $\log_{10}$ *PFU/ml.*
Original tissue culture fluid	8.5	7.7
After purification by Carbowax, chromatography, and ultracentrifugation	10.3	9.2

1.8 over the whole procedure is in excellent agreement with the concentration data, and that very little virus has been lost. In the case of AO-poliovirus with a log increase in titer of 1.5 after concentration, there may have been a small amount of photodynamic inactivation during the course of the purification and concentration procedure.

Staining properties of purified RNA virus concentrates. The final poliovirus concentrates, when fixed and stained with acridine orange, presented the brilliant flame-red fluorescence characteristic of RNA. These fixed preparations were completely susceptible to RNAase indicating that only RNA material was present. No other staining colors were visible in any of these preparations. The TMV concentrates also showed brilliant RNA staining, in the fixed state, again susceptible to RNAase.

A summary of the purified RNA virus staining properties with acridine orange is shown in Table 4. Of particular interest are the results obtained with the fluorescence microscope using unstained concentrates. While poliovirus and TMV exhibited no staining at all under these conditions, the AO-poliovirus concentrates fluoresced a brilliant apple-green, indicating directly that acridine orange fluorochrome had been incorporated into the virus particle. After alcohol fixation, staining was even more pronounced, indicating that dye binding was not just a surface phenomenon in which

the strongly basic acridine orange molecules might be bound to the surface of the negatively charged virus particles. Such an effect would be expected to be nullified by alcohol fixation.

RNA viruses, such as TMV or poliovirus, fluoresce with a brilliant red, but poliovirus grown in the presence of the fluorochrome displays a unique, brilliant, apple-green fluorescence. It is known that basic dyes, such as acridine orange, are bound to nucleic acids by ionic bonds to the phosphate groups, and possibly by weaker Van de Wall's forces. If dye molecules

TABLE 4. STAINING PROPERTIES OF RNA VIRUS CONCENTRATES

	Poliovirus		*TMV*		*AO-Poliovirus**	
Stain	*Unfixed*	*Fixed*	*Unfixed*	*Fixed*	*Unfixed*	*Fixed*
None	No Fluorescence	No Fluorescence	No Fluorescence	No Fluorescence	Brilliant Apple Green	Brilliant Apple Green
.01% A.O.	Pale Green	Brilliant Flame Red	Brilliant Apple Green	Brilliant Flame Red	Brilliant Apple Green	Brilliant Flame Red
.001% A.O.	Pale Green	Brilliant Apple Green	Brilliant Apple Green	Brilliant Apple Green	Brilliant Apple Green	Brilliant Apple Green

* Grown in the presence of 0.0005% acridine orange.

were in proximity to the sugar surface of a nucleic acid molecule, this is sterically possible. So too, in considering the incorporation of acridine orange into maturing poliovirus particles concentrations of the dye in the medium must be taken into account. Schümmelfeder[28] has shown that for fixed tissue at high concentrations of dye (0.05%), cells demonstrated in all parts a change from green→ yellow→ orange→ red with rising pH. With sufficiently low concentrations (0.01%) the cytoplasm (RNA) alone showed a change to red, the nucleus (DNA) remaining yellow even at high pH. The bright green fluorescence of our incorporated concentrates with an RNA virus indicates a low concentration of the dye. We can also conclude that the steric configuration of the RNA molecule makes it possible for larger quantities of the fluorochrome to become attached during the staining process, leading to the development of red fluorescence while under standard conditions the DNA molecule, only, is capable of binding sufficient dye to yield yellow fluorescence.

When the virus concentrates were stained with 0.01% acridine orange at pH 3.8 according to the standard procedure, the following results were obtained. Fixed preparations of poliovirus yielded the brilliant red fluorescence characteristic of RNA; on the other hand, unfixed ones gave the very faintest green indicating either surface binding of the strongly positively charged fluorochrome to the protein of the unfixed virus surface, or limited penetration of the virus particle by the dye. Fixed preparations of TMV also showed red RNA staining, while unfixed preparations, when allowed to remain in contact with the dye solution for some hours, showed a green fluorescence identical to that of the unstained AO-poliovirus. It would appear that, in contrast to poliovirus, the unfixed TMV particle is permeable to acridine orange and that a sufficient concentration of the dye can accumulate within the particle to develop a green fluorescence. The brilliant green color exhibited by the "vitally stained" AO-poliovirus in the unfixed state was not altered by subsequent staining with 0.01% acridine orange. This would be the expected result if the mature, unfixed poliovirus particle could not be penetrated by the fluorochrome. However, the fixed, and subsequently stained, AO-poliovirus concentrates did exhibit a flame-red, RNA fluorescence, as would be expected. These results are listed in Table 4.

The results obtained when staining was carried out with 0.001% acridine orange are also shown in Table 4. With this lower concentration of stain it was impossible to develop the characteristic red RNA color seen with standard staining conditions (0.01%AO) even in fixed preparations. This concentration effect is of particular interest when one considers that optimal conditions for the growth of AO-poliovirus *in vitro* require a concentration of fluorochrome as low as 0.0005%. At this low dye concentration it is impossible to grow mature, infective AO-poliovirus particles exhibiting a typical red RNA fluorescence.

Behavior of RNA virus concentrates after treatment with ribonuclease. The specificity of the RNA staining of all the concentrates studied was established by digestion with ribonuclease. Fixed preparations of poliovirus, AO-poliovirus and TMV were readily susceptible to the enzyme while control virus specimens retained their ability to develop a red fluorescence on subsequent staining. Dried, unfixed preparations of TMV were completely insusceptible to RNAase activity while similar poliovirus preparations were usually susceptible. This is to be expected when one considers the extreme lability of the dried poliovirus particle.

The behavior of fixed preparations of AO-poliovirus with ribonuclease provided additional strong direct evidence that the incorporated vital dye

is, in fact, associated with the nucleic acid moiety of the developing virus particle. The apple green fluorescence of these preparations was almost completely extinguished after incubation with RNAase. This effect was not destroyed by proteolytic enzymes and incubation of similar preparations in the vehicle alone had no effect on these concentrates.

Permeability studies on poliovirus and tobacco mosiac virus. It was possible to carry out a "titration" of the virus surface permeability by staining with acridine orange after fixation in various concentrations of methanol. In the case of poliovirus, fixation and penetration were complete, and red staining developed with concentrations of methanol greater than 70%. Below this methanol concentration, denaturation of the protein coat was not sufficiently extensive to permit penetration of the fluorochrome, and no staining developed. The methanol end point for TMV was slightly lower than that for poliovirus but the general effect was the same. Our experiments with acridine orange demonstrate directly that this vital dye is incorporated into the virus particle. Removal of the characteristic staining with ribonuclease indicates that the dye is, indeed, bound to the nucleic acid of the virus.

As concentrations of acridine orange in excess of 0.003% were highly toxic to tissue culture cells even in the dark, our experiments were carried out in very dilute dye solutions (0.0005%). Even if all of the available dye were to react with the polynucleotide chains of developing virus particles, only minute quantities of dye, incapable of giving a typical red RNA color, would be present despite large aggregates of close-packed particles. Also, from purely physical considerations, it is unlikely that the developing poliovirus particle could incorporate sufficient dye to exhibit other than a green fluorescence. The large quantities of dye required to yield red fluorescence would probably preclude the formation of mature, infective virus particles because of steric hindrance. It would not be possible to form a protein coat around acridine orange-RNA aggregates of such great size. A comparison can be made with TMV, which is definitely permeable to acridine orange. The unfixed TMV particle, with protein coat intact, is penetrable only by sufficient dye to develop a green fluorescence. Hiatt[14] has inferred by direct means that many viruses (e.g., T_3 phage, vaccinia, rabies) are permeable to vital dyes while others (enteroviruses, reoviruses) are completely impermeable. His interpretations are in complete agreement with the results of our direct staining.

Bacteriophage ΦX174, (*Single-Stranded DNA*). Recently there has been a growing interest in a group of small bacterial viruses of which ΦX174 is a member. Hall, *et al.*[24] examined ΦX174 in the electron

microscope and found tail-less particles of 248 Å diameter. They had polygonal outlines consistent with the icosahedral symmetry postulated for many "spherical" plant and animal viruses. Tessman[25] studied inactiviation rates of the same virus by the decay of incorporated radioactive P-32 atoms and obtained an efficiency of inactivation ten-fold greater than those observed with other bacteriophages. He was of the opinion that this indicated the presence of an unusual DNA, possibly a single-stranded form. Sinsheimer[26,27] carried out extensive physical and chemical studies on highly purified preparations of the virus particle and its DNA. The DNA reacted rapidly with formaldehyde, exhibited high flexibility in light scattering experiments, and lacked a complementary nucleotide structure. These findings and the kinetics of enzymatic degradation (by pancreatic deoxyribonuclease) were all consistent with the existence of a single-chain polymer.

We had concluded, from the experiments discussed above, that dye-binding was a matter of steric-fit, and that the color of the emissions from stained viruses depended on dye concentration, not on the chemical differences between nucleic acids. Because the color-shift is not a function of chemical composition, it seemed worthwhile to study the staining reaction of the anomalous nucleic acid in ΦX174.[28] Chemically, it is DNA but its single-strand configuration endows it with unusual physical properties. In our experience two bacteriophages were studied, ΦX174 and T_2. Droplet preparations were fixed, stained with 0.01% acridine at pH 3.8, and examined in blue-violet light. Both DNAase and RNAase were used to check the specificity of staining reactions, and control specimens were incubated in the vehicles for the enzymes.

RESULTS

ΦX174 fluoresced a brilliant flame-red in dried droplet preparations. The fluorescence was destroyed completely when fixed preparations were subjected to DNAase digestion before staining but not when RNAase was used. Incubation of specimens in the enzyme vehicle alone, had no effect on the development of the red fluorescence. Samples of purified T_2, processed at the same time, yielded the typical yellow-green fluorescence of standard DNA.

DISCUSSION

The acridine orange staining properties of bacteriophage ΦX174 differ from those of any other virus previously studied by this method. Red fluorescence at pH 3.8 is usually associated with RNA. In the case of

ΦX174, however, the red fluorescing substrate was destroyed by DNAase but not by RNAase. The apparent paradox of a red-fluorescing DNA is suggestive of a DNA molecular configuration similar to that of RNA, namely, a single-stranded DNA.

It is not necessary to subject preparations of ΦX174 to pre-treatment with a proteolytic enzyme for DNAase to be effective. This is in agreement with the behavior of large bacterial viruses and in contrast to that of the DNA animal viruses in which a close association between the nucleic acid of the virion core and the protein capsid appears to exist.

TABLE 5. STAINING PROPERTIES OF VIRUSES

Virus preparation nucleic acid	*Color developed on acridine orange staining*	*Nuclease susceptibility*		
		DNAase	*RNAase*	*Proteolytic enzyme necessary*
Double-stranded bacteriophage DNA	yellow green	+	—	—
Double-stranded animal virus DNA	yellow green	+	—	+
Animal virus RNA	flame red	—	+	—
Single-stranded bacteriophage DNA	flame red	+	—	—

Schümmelfeder *et al.*[30] reported that concentration of the dye played an important part in the development of fluorescence and that the highly polymerized condition of the DNA molecule prevented intracellular DNA structures from developing a red color, even under conditions of favorable pH. They noted, also, that DNA structures tended to form reddish-fluorescing complexes after acid hydrolysis. We have stressed the importance of the steric arrangement of the DNA and RNA molecules and suggested that RNA should have many more exposed sites for the attachment of acridine orange molecules, a condition favoring the developing of red fluorescence.[15] Similar steric possibilities must exist in the case of the DNA of bacteriophage ΦX174. It would appear that the acridine orange technique, controlled by suitable nuclease digestion tests, is a simple and direct method of detecting DNA in the single-stranded form. It may have applications at the cellular level of virus reproduction.

The existence of a single-stranded DNA molecule as an entity, as in bacteriophage ΦX174, focuses attention on the general mechanisms of DNA replication. Recent results with bacteria and plants are in agreement with the concept of DNA replication by separation of the two chains of the double helix. Whether this results in a complete separation of the strands to a single-stranded state and formation of separate templates is still a matter for speculation; however, it is known that the single-stranded ΦX174 is an excellent primer for the synthesis of a double-stranded DNA with the enzyme *E. coli* polymerase.[30] The fact that

TABLE 6. CsCl ISODENSITY PURIFICATION OF POLYOMA AND ADENOVIRUS

	*Polyoma virus**			*Adenovirus***	
Virus material	*Volume ml.*	*HA/ml.*	*PFU/ml.*	*Volume ml.*	*TCD_{50}/ml.*
Original fluid	30	160	10^{7}	80	$10^{8.5}$
Pellet	1.5	640	$10^{7.4}$	1.5	$10^{5.5}$
CsCl band	1	80	$10^{6.4}$	1	$10^{5.0}$

* Assays kindly carried out by Dr. Matilda Benyesh-Melnick.
** Assays kindly carried out by Mr. E. Seidel.

ΦX174 multiplies readily in *E. coli* yielding infective single-stranded virus particles would presuppose a single→ single-stranded mechanism for DNA replication. From the above results and those of a number of other laboratories, it is possible to set up a simple table to serve as a means for identifying the type of nucleic acid present in different viruses using their staining reactions with acridine orange (0.01% pH 4) and their behavior with specific enzymes (Table 5).

Polyoma Virus and Adenovirus (DNA). Recently attention has been focused on the nucleic acids of polyoma virus[31, 32] and adenovirus.[33, 34] The bulk of experimental evidence indicates that both are DNA viruses. In addition, cytochemical studies with polyoma[35, 36, 37] and adenovirus[38, 39, 40] show that a marked increase occurs in nuclear DNA during the replication of these viruses but it is not known how much of the DNA is actually virus, or if it exists in the single or double-stranded form.

We have purified both viruses by CsCl isodensity centrifugation and have studied the staining reactions of preparations with known virus activity (Table 6). Droplet preparations for acridine orange staining and nuclease digestion tests were made from the CsCl bands dialysed free from the salt. It was a simple matter to place both polyoma and adenovirus

in their correct position in Table 5. The behavior of both viruses was consistent with that of double-stranded, animal virus DNA. It may be argued that any DNA virus replicating in the nucleus could adsorb sufficient cellular DNA to its surface to give a double-stranded color reaction in the standard acridine orange test. However, an additional pre-fixation treatment with DNAase which would certainly remove any contaminating cellular DNA from the preparations, did not interfere with the subsequent development of the specific fluorescence.

This technique of staining CsCl—banded viruses could become a relatively simple test for determining the type of nucleic acid present in known viruses as well as in new agents. Determining information of the nucleic acid in a given virus is required for purposes of classification and for improving our understanding of virus replication.

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