The Fluorescent Staining of Bacteriophage Nucleic Acids

By D. E. BRADLEY

*Department of Zoology, University of Edinburgh, West Mains Road,
Edinburgh, 9, Scotland*

(Received 13 January 1966)

SUMMARY

The staining of viral nucleic acids with acridine orange and their subsequent examination under the fluorescence microscope permits the determination of the type and strandedness. Since the existing procedures are complicated, modifications have been devised which simplify them and avoid the use of the fluorescence microscope, an ordinary ultraviolet lamp being substituted. Also, it has been found that certain post-staining treatments cause colour changes which are related to the strandedness and type of nucleic acid and hence are valuable confirmations of the normally used nuclease sensitivity tests. The new procedures were tested for a wide range of specimens including double-stranded ribonucleic acid, but emphasis has been placed on the identification of bacteriophage nucleic acids.

INTRODUCTION

In the preliminary characterization of a virus or bacteriophage it is important to ascertain the type of nucleic acid which it contains. Nucleic acid analysis is a long and involved process, and clearly a simple identification test would be extremely valuable. The nearest approach to this is the use of the fluorescent stain, acridine orange (Mayor & Hill, 1961; Mayor & Melnick, 1962). With this most valuable technique, it is possible by means of colour differences to differentiate between double-stranded deoxyribonucleic acid (2-DNA) on the one hand and single-stranded deoxyribonucleic acid (1-DNA) or single-stranded ribonucleic acid (1-RNA) on the other. The latter nucleic acids can be differentiated by their respective sensitivities to deoxyribonuclease (DNase) and ribonuclease (RNase). While the procedures of Mayor & Hill, (1961) and Mayor & Melnick, (1962) are simple as compared with the techniques of biochemical analysis, they involve a fairly large number of treatments and the use of a fluorescence microscope. Also, Mayor & Hill (1961) do not differentiate between 1-DNA and 1-RNA by using the stain alone. Furthermore, the colour of acridine orange staining is particularly sensitive to the pH value, so that some difficulty can be experienced in obtaining the correct colours. For these reasons some modifications seemed desirable, particularly the elimination of the fluorescence microscope, which might not always be available.

The method of Mayor & Hill (1961) involves the following steps. A virus suspension is dried down in droplets at the centres of coverslips, which are then treated with Carnoy's fixative for 5 min. They are next hydrated in an ethanol series and rinsed in distilled water. After treatment in McIlvaine's citric acid + phosphate buffer at pH 4.0 for 10 min., the coverslips are stained in 0.01 % (w/v) acridine

orange in McIlvaine's buffer at pH 4.0 for 5–10 min. They are then examined in a fluorescence microscope. The colours obtained are yellow–green for 2-DNA viruses and flame-red for 1-DNA and 1-RNA viruses. The modifications described in the present paper are the substitution of direct viewing under ultraviolet (u.v.) radiation instead of the use of the fluorescence microscope, the simplification of the pre-staining treatment, the elimination of possible unreliability due to pH-sensitivity arising from this, and the addition of a post-staining treatment to differentiate 1-DNA and 1-RNA bacteriophages.

The test specimens used here consisted mostly of bacteriophages, but also included 2-RNA from plant wound tumour virus and the intact 2-RNA reovirus. The differentiation of 2-RNA and 2-DNA is considered in some detail since it will be of importance in facilitating the discovery of a 2-RNA bacteriophage.

METHODS

The following simplified procedure for acridine-orange staining is basically similar to that described provisionally by Bradley (1965). The treatments after staining were evolved by trial and error, by using known types of nucleic acids and numerous reagents.

Table 1. *Test specimens of various viruses*

Test specimen	Nucleic* acid	Source of preparation†	Approximate concentration
Phage T4	2-DNA	Lark	1×10^{11} particles/ml.
Phage E1‡	2-DNA	Author (broth lysate)	7×10^{10} particles/ml.
Mouse liver DNA	2-DNA	Walker	0.1 % (w/v)
Mouse liver DNA (denatured)	1-DNA (near)	Walker	0.025 % (w/v)
Phage ZJ/2	1-DNA	Kay	1×10^{12} particles/ml.
Phage ØR¶	1-DNA	Author (lysed plates)	5×10^{11} particles/ml.
Phage ZIK/1	1-RNA	Author (broth lysate)	5×10^{12} particles/ml.
Phage 7s§	1-RNA	Author (lysed plates)	5×10^{12} particles/ml.
Tobacco mosaic virus	1-RNA	Babos	0.5 % (w/v)
Wound tumour virus RNA	2-RNA	Black	0.04 % (w/v)
Reovirus	2-RNA	Spendlove & Lennette	1×10^{10} particles/ml.

* Nucleic acids: 1-, 2-, single-stranded, double-stranded.

† For addresses of suppliers, see acknowledgements.

‡ Bradley, 1963. || Bradley, 1964. ¶ Kay 1962.

§ Phage 7s is a *Pseudomonas aeruginosa* phage (Feary, Fisher & Fisher, 1964) and the other phages are coliphages.

Preparation of test specimens. The test specimens and their concentrations are listed in Table 1. The bacteriophages were prepared by standard plating or broth culture methods. Crude phage suspensions were treated with DNase and RNase at a concentration of about 30 $\mu\text{g.}/\text{ml.}$ for 1–2 hr at 37°. Purification was by alternate high-speed and low-speed centrifugation. Mouse liver DNA was used in a weak saline solution. It was also denatured to make it resemble 1-DNA, as follows. About 0.5 ml. was placed in a test tube with a pipette and the test-tube immersed in a

beaker of boiling water for 5 min. It was then quickly transferred to an ice and salt bath where it was rapidly agitated until frozen. On thawing, it was diluted to 0.025 % (w/v) with phosphate-buffered saline (Na_2HPO_4 , 1.27 g.; KH_2PO_4 , 0.41 g.; NaCl , 7.36 g.; per l. distilled water; pH 7.2). Tobacco mosaic virus was concentrated from a suspension by centrifugation and resuspended in phosphate-buffered saline.

Pre-staining fixation. Small droplets of the specimen suspension (0.002–0.005 ml.) were placed on microscope slides and dried in a stream of warm air. When subsequent staining showed that the concentration of a specimen was too low, further droplets up to a total of four were dried on top of the existing ones (this was necessary with the reovirus). The resulting 'spots' were fixed in Carnoy's fluid (1 part glacial acetic acid; 3 parts chloroform; 6 parts ethanol) or methanol as follows. For Carnoy fixation the slides were placed in a Petri dish containing the fluid for 5 min. at room temperature. They were then removed, washed briefly in absolute ethanol and dried in a stream of warm air. For methanol fixation, the slides were placed in a dish of chilled methanol for 15–20 min. at 2°. They were then removed and dried in warm air.

Acridine-orange staining and post-stain treatments. The following reagents were prepared:

Modified McIlvaine's citric acid + phosphate buffer, pH 3.8: 0.1 M-citric acid, 6.0 ml.; 0.15 M- Na_2HPO_4 , 4.0 ml.

Acridine orange 0.01 % (w/v) in buffer: 1 % (w/v) acridine orange solution, 0.1 ml.; modified McIlvaine's buffer, 10.0 ml.

Disodium hydrogen phosphate (Na_2HPO_4); 0.15 M.

Molybdic acid solution: molybdic acid (MoO_3), 1 g. boiled for 1–2 min. in 100 ml. water and cooled. The solution was decanted from the precipitate and diluted 9:1 before use.

Tartaric acid, 0.1 M.

Acridine-orange staining and subsequent treatments were done in the following sequence. (1) The dried fixed slides were placed in 0.01 % (w/v) acridine orange in modified McIlvaine's buffer at pH 3.8 for 5 min. (2) They were rinsed twice, briefly, in two separate baths of McIlvaine buffer at pH 3.8. (3) The slides were soaked in 0.15 M-disodium hydrogen phosphate solution for 15 min. (4) Excess liquid was shaken off and the colours of the spots observed under u.v. radiation, wave-length 2537 Å, from a portable u.v. lamp (low pressure mercury tube, Hanovia Lamps Ltd., Slough). This treatment indicated whether the virus contained 2-DNA or 2-RNA on the one hand, or 1-DNA or 1-RNA on the other (see Results).

Further differentiation was achieved as follows. (5) A dish of molybdic acid solution was placed beneath the u.v. lamp. (6) The slide from (4) above was dipped in and out of the solution, the colour changes being continuously observed. The time required for the completion of these changes was between 15 sec. and 90 sec. The spots of double-stranded nucleic acids remained the same colour, but those of the single-stranded types changed as described in the results, thus permitting their differentiation.

Confirmation of the results was obtained as follows. (7) A dish of 0.1 M-tartaric acid was placed beneath the u.v. lamp. (8) Another slide from (4) above was placed in the solution. Colour changes were observed over 2–5 min. without removing the slide from the solution, which is transparent to u.v. radiation.

Ribonuclease and deoxyribonuclease digestion tests. As recommended by Mayor & Hill (1961) RNase and DNase digestion tests were a valuable confirmation of results obtained with staining. Because of the difference in the present staining procedure and some difficulties encountered with DNase treatment under neutral conditions, alternative methods of enzyme treatment were worked out. Digestion with ribonuclease (RNase) was done as follows. (1) Carnoy-fixed or methanol-fixed spots (two of each specimen) were soaked in modified McIlvaine's buffer (pH 3.8) for 5 min. (2) A slide with one spot was removed and placed in 0.1 % (w/v) RNase in buffer at pH 3.8. The second spot, the control, was left in buffer alone. (3) The dishes of both buffer and RNase solution were incubated at 37° for 2 hr. (4) Both control and treated spots were then stained in acridine orange in the normal way. (5) Colours were observed under u.v. irradiation before and after Na₂HPO₄ treatment as described above (Section 3, acridine-orange staining).

Spots which are susceptible to RNase, i.e. which give no colour after treatment, as compared with the control spot, will contain RNA and those resistant will contain DNA, with the exception of spots containing 2-RNA.

Digestion with deoxyribonuclease (DNase). Mayor & Hill (1961) used DNase in a neutral veronal buffer for treating spots but, in the present work, the spots dissolved under such conditions. It was also noted that the wax pencil marks used for marking the slides floated off in neutral but not in acid solutions. It was concluded that the same was happening to the spots and that DNase treatment would have to be carried out in acid conditions to avoid losses. Since citric acid is reputed to inactivate DNase, and the optimum pH value for the operation of the DNase is near neutral, a mildly acid phosphate + acetate buffer was chosen and found to be satisfactory. This was prepared as follows: a 0.15 M solution of Na₂HPO₄ was brought to pH 5.5 with 10 % (w/v) acetic acid; magnesium was added as acetate to a concentration of 0.003 M to improve the efficiency of the DNase.

DNase digestion was done as follows. (1) Two spots were fixed and dried as for RNase above. (2) They were soaked in phosphate + acetate buffer (pH 5.5) for 5–10 min. (3) A slide with the spot to be treated was removed and placed in a dish of 0.02 % (w/v) DNase in phosphate + acetate buffer. (4) Incubation of the control and DNase baths was carried out at 37° for 2 hr. (5) After removal, the slides were soaked in modified McIlvaine's buffer (pH 3.8) for 5–10 min. and then stained as described above. (6) Colours were observed under u.v. radiation before and after treatment with Na₂HPO₄ solution. As with the RNase digestion, appropriate susceptibility to DNase indicated the type of nucleic acid present.

With the procedures outlined above, the type of nucleic acid contained in a bacteriophage can be definitely established with a very small quantity of suspension in a comparatively short time.

RESULTS

Colours obtained by acridine-orange staining

Staining and enzyme tests were done at least three times on each test specimen; reproducible results were obtained in all cases. The colours obtained are given in Table 3. Since the colours observed immediately after staining and without disodium hydrogen phosphate treatment were often a mixture of red and green, and consequently meaningless, they are not recorded. A description of the actual colours

obtained by the procedure described here is necessary since the classical description is somewhat misleading. The green of 2-DNA and 2-RNA has been described as 'apple- or yellow-green'. This is close to its appearance, the shade being rather yellower than, for example, a barium-coloured flame, but bluer than a molybdenum-coloured flame; it is in fact between the two. The red of 1-DNA and 1-RNA is generally called 'flame-red'. This is misleading since impressions of 'flame-coloured' vary widely from person to person. The colour is not far from crimson, slightly deeper than the red of the sunlight spectrum, and close to the red of strontium-coloured or lithium-coloured flames. An orange colour is obtained after the tartaric acid treatment of 2-DNA; this is near the true orange of the sunlight spectrum and is slightly redder than that of orange fruit. Sometimes the red of 1-RNA changes hue after the molybdic acid treatment to a much deeper crimson.

Table 2. *Colours of stained test specimens*

Specimen*	Nucleic acid	After Na_2HPO_4	molybdic acid	After tartaric acid
T4	2-DNA	Bright green	Bright green	Bright green
E1	2-DNA			
ML DNA	2-DNA			
ML DNA (denatured)	1-DNA (near)	Bright red	Paler green	Bright red
ZJ/2	1-DNA	Bright red	Paler green	Paler green
ØR	1-DNA	Bright red	Paler green	Paler green
ZIK/1	1-RNA	Bright red	Paler red	Bright red
7s	1-RNA	Bright red	Paler red	Bright red
TMV	1-RNA	Bright red	Paler red	Pale green
WTV-RNA	2-RNA	Bright green	Green (fades)	Pale red
Reovirus	2-RNA	Green	Green (fades)	Pale red

* See Table 1; abbreviations: ML = mouse liver, TMV = tobacco mosaic virus, WTV = wound tumour virus.

Table 2 shows that the colours obtained are remarkably consistent with two exceptions. First, it can be seen that denatured mouse liver DNA did not behave with tartaric acid like the 1-DNA of bacteriophages. The probable reason for this is that denatured 2-DNA is not identical with natural 1-DNA. Secondly it will be noted that tobacco mosaic virus gave a pale green colour after tartaric acid treatment; the 1-RNA of bacteriophages remained bright red. It is thought that the reason for this is because tobacco mosaic virus has a very high protein:nucleic acid ratio as compared with bacteriophages; excess protein can produce a background green colour which may mask the red.

Susceptibility to nucleic acid enzymes

The results obtained with the test specimens are given in Table 3. It is important to compare the colour obtained with the control spot with that of the treated spot. With a susceptible spot, there should be a very much less intense colour or a complete absence of colour. It may be argued that with the enzyme tests, all that is required of acridine-orange staining is that it should produce any colour, to indicate a positive or negative result, so that the phosphate treatment is unnecessary.

However, it is considered important to be certain that the staining procedure has worked correctly, a result indicated by obtaining the correct colour for the type of nucleic acid after phosphate treatment. For this reason, colours obtained without phosphate treatment in the enzyme tests, which are invariably a mixture of red and green, have not been recorded.

It will be noted in Table 3 that the ØR spot was not completely removed with DNase. While testing various phage suspensions prepared by plating methods, it was found that the presence of a large amount of proteinaceous debris or agar could completely inhibit the action of nucleic acid enzymes on a spot, presumably because the overlying material prevented access of the enzyme to the nucleic acid molecules.

Both Carnoy fixation and methanol fixation were tested for in the staining and enzyme digestion reactions; essentially similar results were obtained. While the difference was marginal, enzyme digestion seemed to be more efficient after methanol fixation, and staining after Carnoy fixation.

Table 3. *Susceptibility to RNase and DNase*

The colours given are those obtained after treatment in 0.15 M Na_2HPO_4 solution for 15 min. after staining.

Specimen*	Nucleic acid	RNase		DNase	
		Control	RNase	Control	DNase
T4	2-DNA	Bright green	Bright green	Bright green	Nil
E1	2-DNA				
ML DNA	2-DNA				
ML DNA (denatured)	1-DNA (near)	Bright red	Bright red	Bright red	Nil
ZJ/2	1-DNA				
ØR	1-DNA	Bright red	Bright red	Bright red	Pale red
ZIK/1	1-RNA	Bright red	Pale green	Bright red	Bright red
7s	1-RNA	Bright red	Nil	Bright red	Bright red
TMV	1-RNA	Bright red	Pale green	Paler red	Paler red
Reovirus	2-RNA	Pale green	Pale green	Pale green	Pale green

* See Table 1; abbreviations: ML = mouse liver, TMV = tobacco mosaic virus.

Table 4. *Colours for different types of nucleic acid*

Nucleic acid	Treatment				
	Na_2HPO_4	Molybdic acid	Tartaric acid	RNase	DNase
2-DNA	Green	Green	Orange	— *	+
2-RNA	Green	Green (fading)	Red	—	—
1-DNA	Red	Paler green	Paler green	—	+
1-RNA	Red	Paler red	Paler red	+	—

* Resistance to an enzyme is denoted by —; susceptibility is denoted by +.

Summary of results

The results on the test specimens may be summarized according to nucleic acid type; in Table 4 the colours for each are given together with appropriate enzyme reactions.

DISCUSSION

In order to obtain unambiguous and reproducible results with the tests, one or two points must be emphasized. It is essential to have a reasonably pure virus preparation otherwise incorrect colours or reactions to enzyme digestion may be obtained because of contaminating protein. This must therefore be removed. Alternate high- and low-speed centrifugation is usually adequate but must be done several times with a low-speed value of about 7000g for 20 min. This may cause a slight loss of some larger bacteriophages. With some animal viruses treatment with a proteolytic enzyme is essential.

As has been mentioned, the concentration of the specimen is not critical, but it must be borne in mind that some fading occurs with post-staining reagents, especially molybdic acid. The spots should therefore be of sufficient concentration to give a bright fluorescence before post-staining treatments. However, too much virus can give false colours (e.g. a green edge to the red spot obtained with 1-DNA phages). Conversely too little virus gives faint colours (some difficulty was encountered with reovirus because of this). In general the concentrations given in Table 1 are adequate. With large phages an obvious opalescence in suspensions indicates a correct concentration. With small 1-RNA or 1-DNA types 10^{11} – 10^{12} particles/ml. is ideal. Some viruses with a low nucleic acid content may require as many as 10^{13} particles/ml. Viruses in general are easily prepared in the required amounts; a total volume of 0.1–0.2 ml. at the correct concentration is sufficient for all five tests.

It is also emphasized that Carnoy fixation or methanol fixation is essential in order to render the specimen susceptible to staining and enzyme digestion. Also, unless the correct suspending medium is used for the specimen, staining may be adversely affected and misleading colours may be obtained.

While in general it is advisable to carry out all the tests on a given specimen, it may not always be necessary to do the enzyme digestion reactions where there are other indications of the type of nucleic acid present. For example, in the case of a bacteriophage where RNase inhibits plaque formation on double agar layer plates, and a red colour not changing in molybdic acid is obtained with staining, the nucleic acid is undoubtedly 1-RNA.

Because the results with the tartaric acid post-staining treatment are not absolutely consistent, it is considered that this should be treated as a confirmatory test, particularly for double-stranded nucleic acids where the change from green to orange or red is characteristic. Unfortunately, it has not yet been possible to find a post-staining treatment which will differentiate 2-DNA from 2-RNA. The fading of the green colour in molybdic acid with 2-RNA as opposed to the stability of the green of 2-DNA is not entirely reliable, though it may be a guide. This means that the differentiation is largely dependent upon the susceptibility to enzymes. It should be noted that 2-RNA was resistant to the RNase treatment, an observation contrary to that quoted by Jamison & Mayor (1965). Unfortunately it was not possible to make satisfactory enzyme tests on spots of purified plant-wound tumour virus RNA because the spots dissolved in the control baths of buffer solution. This was also the case with purified bacterial RNA and was presumably due to the lack of protein to stick the short strands of nucleic acid to the slide. However, there is other

evidence to support the result on reovirus. Weissmann *et al.* (1964) showed that the replicating form of the RNA of coliphage MS2 was double-stranded and RNase-resistant. Nonoyama & Ikeda (1964) gave a similar result and Dr D. H. L. Bishop (personal communication) found that 2-RNA isolated from RNA phage-infected *Escherichia coli* cells was RNase-resistant at salt concentrations of about 0.15 M but not at < 0.1 M; it was almost completely digested in 0.01 M salt. In the case of a spot with much crystallized salt, and with more than 0.1 M salt present in the buffered solutions of RNase, 2-RNA will be RNase-resistant. It might, however, be possible to make it RNase-sensitive by decreasing the salt concentration and the magnesium content in the RNase solution. For the purpose of the present tests it is considered to suffice that 2-RNA is DNase-resistant. The action of DNase on 2-DNA spots is so efficient and complete that there can be no room for doubt.

The advantages of the tests proposed here are obvious and they can be applied to any virus. The procedures described are not intended to supersede those of Mayor & Hill (1961), without which it would have been impossible to work out the present ones. The modifications are designed to make the original methods more readily available. This has been achieved at the cost of some sensitivity; nevertheless only small amounts of virus are required. Such tests are important at present since nucleic acid type is being considered as a major taxonomic criterion in the classification of viruses. In conclusion it may be pointed out that tests of this kind are bound to be, to some extent, subjective, especially where the fluorescence is faint. While there is obviously no question about distinguishing green from red, judgment of the colour intensity in enzyme tests may leave room for doubt; the post-staining treatments are therefore an added safeguard. It is recommended that both the molybdic acid and the tartaric acid tests should be used and, when inconsistent results are obtained, the virus suspensions should be repurified. By going through the whole procedure of staining, post-staining treatment and enzyme digestion, and by including control spots of viruses which give the correct colours, much of the subjective element can be removed and it will be obvious whether or not the specimen suspension is sufficiently pure.

The author thanks the following for generously providing samples of nucleic acids, bacteriophages and other viruses (see Table 1). Dr and Mrs K. G. Lark (Kansas State University, Manhattan, Kansas), Dr P. M. B. Walker (Zoology Department, Edinburgh University), Dr D. Kay (Sir William Dunn School of Pathology, University of Oxford), Dr I. Babos (Department of Botany, University of Edinburgh) Professor L. M. Black (Department of Botany, University of Illinois, Urbana, Illinois), Drs R. S. Spendlove and E. H. Lennette (Department of Public Health, Berkeley 4, California). The author is also grateful to Dr D. H. L. Bishop for discussions and to Miss C. A. Dewar for valuable technical assistance.

REFERENCES

- BRADLEY, D. E. (1963). The structure of coliphages. *J. gen. Microbiol.* **31**, 435.
- BRADLEY, D. E. (1964). The structure of some bacteriophages associated with male strains of *Escherichia coli*. *J. gen. Microbiol.* **35**, 471.
- BRADLEY, D. E. (1965). The staining of bacteriophage nucleic acids with acridine orange. *Nature, Lond.* **205**, 1230.
- FEARY, T. W., FISHER, E. & FISHER, T. N. (1964). Isolation and preliminary characteristics of three bacteriophages associated with a lysogenic strain of *Pseudomonas aeruginosa*. *J. Bact.* **87**, 196.
- JAMISON, R. M. & MAYOR, H. D. (1965). Acridine orange staining of purified rat virus strain x14. *J. Bact.* **90**, 1486.
- KAY, D. (1962). The nucleic acid composition of bacteriophage Φ R. *J. gen. Microbiol.* **27**, 201.
- MAYOR, H. D. & HILL, N. O. (1961). Acridine orange staining of a single-stranded DNA bacteriophage. *Virology* **14**, 264.
- MAYOR, H. D. & MELNICK, J. L. (1962). Intracellular and extracellular reactions of viruses with vital dyes. *Yale J. Biol. Med.* **34**, 340.
- NONOYAMA, M. & IKEDA, Y. (1964). Ribonuclease resistant RNA found in cells of *Escherichia coli* infected with RNA phage. *J. mol. Biol.* **9**, 763.
- WEISSMANN, C., BORST, P., BURDON, R. H., BILLITER, M. A. & OCHOA, S. (1964). Replication of viral RNA. III. Double-stranded replicative form of MS2 phage RNA. *Proc. natn. Acad. Sci., U.S.A.* **51**, 682.