A Study of the Negative Staining Process

By D. E. BRADLEY

Department of Zoology, University of Edinburgh, Scotland

(Received 3 February, 1962)

SUMMARY

The effectiveness of a number of different materials in the negative staining method for electron microscopy of viruses is evaluated. Shadowing was used to study the degree of distortion suffered by the specimen. Changes in pH value which occurred while the negative staining solution was drying were measured in those materials which gave the most satisfactory results in the electron microscope. The ultimate resolution of the method is discussed and demonstrated. It was important to de-grease supporting film: perforated carbon films were found valuable for obtaining good contrast.

INTRODUCTION

Negative staining is used in light microscopy to reveal objects which will not take up conventional stains. The specimen is placed in a solution containing black pigment (nigrosin or aqueous indian ink) and is seen transparent against the dark background. The same principle is used for the electron microscope (Hall, 1955; Huxley, 1956; Brenner & Horne, 1959) but the preparation is in a solid, not a liquid state. The method consists of embedding an electron-transparent object in a structureless electron-dense matrix. The most widely used matrix is potassium phosphotungstate; the normal procedure is to mix a suspension of the specimen in water or ammonium acetate with a solution of phosphotungstate, and to apply this to carbon-coated grids in one of two ways. The mixture can be sprayed (Brenner & Horne, 1959) or spread by allowing a thin film of liquid to dry down on the grid (Bradley & Kay 1960; Huxley & Zubay, 1960). The results obtained with these methods have been valuable and have contributed much to our knowledge of the morphology of small particulate specimens, especially viruses. However, relatively little work has been done on the technique itself, and a number of points require elucidation. First, the physical features are not fully understood. These concern the thickness of the matrix, the degree of three-dimensional preservation it provides, and the conditions under which the matrix spreads. Furthermore, little attempt has been made to use other chemicals, it being assumed that phosphotungstate is the most satisfactory. Great emphasis has been placed on using the correct pH value of solutions at the start of the process, but the fact that this may not remain constant has been overlooked. Negative staining mixtures change their pH values as they dry down (Bradley, 1961*a*); this probably affects the specimen to some degree. Though applied mainly to the study of bacteriophages, the present communication attempts to provide facts of general application relating to these points.

METHODS

The spraying and spreading methods of negative staining have their advantages and disadvantages. For example, quantitative virus counts (Dr D. Watson, personal communication) can only be carried out by using a spray, but with the spray the virus concentration is much more critical than with spreading. On the whole, the spreading method is easier, quicker and uses less virus suspension. This technique was therefore used to obtain the results described here.

The preparation of support films

The physical state of the surface of a support film affects the spreading of the negative-staining material into areas of suitable thickness. Thus, Brenner & Horne (1959) found that phosphotungstate did not spread properly on carbon films prepared in a vacuum unit which included an oil-diffusion pump. It is thus important that, particularly when materials are being tested for suitability as negative-staining agents, the support films used should be free from contaminating oil molecules; if consistent results are to be obtained coated grids should be carefully de-greased by dipping the grid into a solvent, e.g. re-distilled chloroform, immediately before use. Most types of carbon support film are generally suitable, even those which consist of a plastic stabilized with carbon.

It is considered that perforated carbon films (for a suitable method, see Bradley, 1961*b*) are best; many holes are covered with embedding chemical (Huxley & Zubay, 1960) and specimen particles are included in them. The contrast obtained under such conditions has been found to be improved, and the background structure seen significantly diminished. The reason for this is that the presence of a support film not only decreases contrast, but also produces a variable background structure which may be as coarse as 20 Å., depending on the nature and condition of the substrate. It has been found that with the spreading method of applying phosphotungstate the thickness of the matrix varies over both holes and grid. This is an advantage because specimen particles embedded under optimum conditions can be readily found and the method used at maximum efficiency.

The preparation and use of negative-staining solutions

Several solutions have been tested as embedding media for negative staining. They were all prepared in the same way except for solutions of ethylenediaminetetra-acetate (EDTA) complexes. Chemicals, analytical reagent grade when available, were used as 1–2% aqueous solutions (concentration not very critical with the spreading method), their pH values being adjusted with potassium or sodium hydroxide for salts of these metals, and with ammonium hydroxide for other salts.

EDTA complex solutions (Van Bruggen, Wiebenga, & Gruber 1960) were prepared as follows: a 1% suspension of ethylenediaminetetra-acetic acid in water was made up. This was mixed with an equal volume of 1% uranyl acetate or nitrate solution. Ammonia was then added until the suspended EDTA disappeared, the pH being finally adjusted to the desired value with ammonia or acetic acid. Solutions containing EDTA were used first, to enable uranyl acetate or nitrate solutions to be raised to alkaline pH values without forming a precipitate, and secondly, to investigate the spreading characteristics of the compound.

Test specimens consisted of various types of bacteriophage (see below) suspended in one of the following solutions (strength 0.1 M, pH about 7.2): ammonium acetate, ammonium carbonate, ammonium benzoate.

The preparation of negative-staining mounts was done as follows. Equal volumes (unless otherwise stated) of virus suspension and negative-staining solution totalling about 0.02 ml. were mixed on a watch-glass or slide by a micropipette. A freshly de-greased support film was then touched on to the surface of the mixture. Excess liquid was removed from the grid with filter paper until only a thin film covered the grid. After drying, the specimen was ready for examination.

Two general practical features are worth mentioning here. First, spreading agents (chemicals which assist the embedding matrix to spread evenly over the support film) in general were found to be unnecessary. However, the following materials have been satisfactorily used in conjunction with phosphotungstate: 0.2–0.4% sucrose + a trace of tryptone (Anderson, 1960); bovine serum albumin trace (Harrison & Nixon, 1960); EDTA (present communication) + uranyl salts only. Secondly, trouble may often be experienced from breaking of the support film, but this may usually be remedied by using the correct support grid. For perforated films, either Smethurst Highlight type A.E.I. or Veco 400-mesh/in. or their equivalents are used. For continuous carbon or plastic + carbon films the standard 200-mesh/in. are generally suitable.

CHARACTERISTICS OF THE SPREADING METHOD

When a thin film of negative-staining mixture dries on a support film, it generally provides a wide variation in concentration of dried material both over the grid as a whole and over individual grid squares. At first sight this may seem to be a disadvantage, but in fact the opposite is the case. A short search invariably shows areas where the staining characteristics are satisfactory, and it is rarely necessary to prepare a fresh grid. The concentration of dried material is usually greater towards one edge of the grid than the other, especially when it has been held vertically during drying. Thus, when it is found that some grid squares are completely 'blacked out' with embedding matrix, it is only necessary to move towards the opposite edge. The distribution of material is usually symmetrical over a grid square, the concentration being highest around the outside and decreasing towards the centre, where the most satisfactory areas are to be found. As Brenner & Horne (1959) stated, satisfactory results depend also on the ratio of specimen particle concentration to embedding material concentration. Where there are no particles, the matrix, depending upon its chemical composition, may aggregate into large dense spheres, and where there are too many particles, contrast may be low. Again, this ratio appears to vary both across grid and grid square, and suitable regions with different particle:matrix ratios can easily be located. These difficulties are encountered mainly with the less successful preparations; extensive areas of the grid are often entirely satisfactory. Thus, though the method is not completely consistent in that the general quality of preparations varies, it is almost certain that good micrographs can be obtained from any one grid. Compared with other electron microscope specimen techniques, this is one of the more reliable, and undoubtedly one of the simplest.

When the spreading method is used, it will be found that on examination in the electron microscope three different embedding conditions are encountered, each of which depends upon local concentrations of specimen particles and embedding medium. First, there is the case when the particle is completely embedded in matrix. Depending on the specimen this may or may not give a good micrograph. Shadowing of negatively stained preparations has revealed that the particles in such areas are often distorted and not always covered completely, but the most usual state of affairs is that shown in the diagram *a* in Fig. 1. This condition is generally considered by most workers to be the most desirable, and also applies when particles are embedded in matrix covering holes. A second condition is when an individual particle is embedded in a small area of matrix as shown in Fig. 1*b*. The contrast-forming conditions differ little from the first case, but it is often found that better

Fig. 1 (*a*). Diagram of particles deeply embedded in a large area of negative staining matrix. (*b*) Diagram of particles embedded in a small area of matrix. (*c*) Diagram of a single 'high and dry' particle with adsorbed layer of matrix.

resolution is obtained. A third condition occurs when particles are left 'high and dry' due to surface tension effects. Here, they generally have a thin film of the matrix adsorbed on their surface as shown in Fig. 1*c*. The resulting contrast is quite different and closely resembles that given by positive staining. Thus it is found that completely different aspects of a particle may be visualized on a single grid square and it is therefore recommended that micrographs should be taken of specimens found under a variety of embedding conditions.

An interesting feature of both spraying and spreading methods has been noted by Dr D. Watson (personal communication). He found that polystyrene latex particles appeared black when not surrounded with phosphotungstate, but white when embedded in matrix on the same micrograph. The difference was real, and could be measured with a densitometer. As yet no explanation has been found for this phenomenon, but Dr Watson has suggested that possibly there might be a phase shift in the electron beam as it passes through the matrix (which may be 500 Å or more thick), producing reversal of contrast. This is a point which must be carefully considered when interpreting micrographs of embedded and non-embedded particles.

Test objects and their structure

The following phages, which were used as test objects, were obtained from the following sources: Phages T2, T5, ØR, 66t- from Dr D. Kay (Sir William Dunn School of Pathology, University of Oxford); phage T4 from Dr and Mrs K. G. Lark (Saint Louis University School of Medicine); staphylococcus phage 70 from Dr Elisabeth Sharpe (National Institute for Research in Dairying, Shinfield, near

Reading). They were all purified by several cycles of centrifugation and used at concentrations between 10^{10} and 10^{11} particles/ml. Phage 1C was isolated in this laboratory.

When investigating a technique it is advisable to use test objects which have been well studied. For this reason, coliphages T2 and T4 were chosen. It was also considered desirable to use a particle similar to small spherical plant and animal viruses which at the same time would provide a severe test for the methods used. Phage ØR was chosen for this (Kay, 1962; Bradley, 1961; Kay & Bradley, 1962).

The general structure of phages T2 and T4 is now well known (Brenner *et al.* 1959). More recently, however, newly discovered features of both phages have aroused interest. On T4 there is a 'thin collar or disc' (Anderson, 1960), and Daems, Van der Pol & Cohen (1961) detected an 'outer jacket'. The present author (in preparation) has studied these features and found that on T4 they consist of an open-mesh outer sheath of protein fibres attached at the top to the collar. Such features are shown in Pl. 1, fig. 6, on a different but morphologically similar phage (66t-). T2 is similar except that the collar is absent and the fibres appear to be attached to the base of the head (Pl. 1, fig. 4). This fibrous structure, together with the familiar cross-striations as well as the relative instability of the extended sheath, provide excellent delicate features to test preservation.

Bacteriophage ØR consists of a core of single-stranded DNA surrounded by a protein coat in the form of an icosahedron 300 Å in size. There are small subunits at its apices, apparently in the form of dimers. The presence or absence of these subunits is a valuable guide to the qualities of preservation provided by a negative stain, since they are small and easily lost.

Coliphage T5 and staphylococcus phage 70 have also been used and will be described in the appropriate section.

The investigation of different embedding chemicals

Potassium phosphotungstate (PTA). Because this chemical has been the most widely used and is considered the most efficient, it was studied under a variety of conditions. Phages T2, T4, 66t- and ØR were used as test objects. The mixtures of the three buffer solutions already mentioned with phosphotungstate were tested under neutral conditions. On the whole, a mixture of 0.1 M-ammonium benzoate and 2% phosphotungstate provided good spreading, contrast and preservation with phage T4 (Pl. 1, figs. 1, 2), but some sort of chemical reaction appears to have occurred with the T2 sheath protein causing it to contract and disrupt (Pl. 1, fig. 3). The fibrous sheath is broken up to some extent in both cases.

With 0.1 M-ammonium carbonate no such chemical action occurred with phage T2, and preservation seemed, if anything, slightly better. In Pl. 1, fig. 4, the fibrous sheath appears to be intact, albeit lacking a little in contrast. In phage T4 the collar and slightly disrupted fibres are clear in Pl. 1, fig. 5.

With 0.1 M-ammonium acetate, much the same state of preservation is obtained. Phage 66t-, which is morphologically similar to phage T4, was often found perfectly preserved (Pl. 1, fig. 6). Though the phosphotungstate + ammonium acetate mixture appeared to be rather better in this series, it is not considered to be significantly superior to either of the other combinations. However, care must be taken in interpreting micrographs taken when using ammonium benzoate because of the

risk of the chemical action mentioned above. For preparing specimens, therefore, there is little to choose between these three mixtures, as all of them produced excellent results, save for the rather poor preservation of the large heads.

An exceptionally good (Pl. 2, fig. 7) and an average (Pl. 2, fig. 8) micrograph of phage ØR in phosphotungstate + ammonium acetate are shown. The best was taken of a preparation spread over a hole; the advantages of this are immediately obvious. These may be compared with other micrographs obtained when different mixtures described below are used.

The shadowing of phosphotungstate preparations indicates the thickness of the matrix, which is generally about 500 Å. In addition, as shown in Pl. 2, fig. 9, the state of preservation of the heads can be seen; they have completely collapsed. This appears to be the one undesirable feature of phosphotungstate, though it does not always happen. Care should be taken in interpreting asymmetric structures seen in large bodies as real, because there is a very great risk of artifact formation.

Sodium tungstate. Results obtained with sodium tungstate under neutral conditions + ammonium acetate are comparable with results with phosphotungstate. The apical subunits of phage ØR are clear in Pl. 2, fig. 10, though in other micrographs they show a tendency to fall off. With the specimen suspended in ammonium carbonate or benzoate the results were unsatisfactory showing poor definition and crystallization, respectively. Sodium tungstate is considered a reliable addition to potassium phosphotungstate.

Sodium phosphomolybdate (PMA), was tested under neutral conditions. With the phage in ammonium benzoate, there was crystallization; when in ammonium carbonate most phage T4 tails were disrupted (Pl. 2, fig. 11), the fibrous outer sheath lost, but the head shape well preserved. Improved preservation was obtained with ammonium acetate (Pl. 2, fig. 12), but the contrast was rather low and, as a result, the support film background structure was more noticeable. The results with phage ØR were not good (Pl. 2, fig. 13), the apical knobs being scarcely visible. Very good definition has been obtained with phosphomolybdate in the past, and if it could be spread across holes—a difficult thing to achieve at present—excellent results might be obtained.

Sodium molybdate. Under neutral conditions this chemical crystallizes with ammonium benzoate, and rather poor results were obtained with ammonium acetate with phage T4 (Pl. 3, fig. 14) and phage ØR (Pl. 3, fig. 15). However, with ammonium carbonate, preservation and contrast were greatly improved and the matrix spread across holes. This mixture has only been studied with phage T4, but was found as good as any of the phosphotungstate mixtures (Pl. 3, fig. 15).

Uranyl acetate. Unfortunately this important and useful chemical can only be used in acid (pH 5.2) solution. Its spreading characteristics are unreliable but with ammonium acetate it frequently behaves like phosphotungstate. Shadowing revealed incomplete wetting of the phage particles, phage T2 being the example (Pl. 3, fig. 17; see tail of bottom particle). Particles were also found with a thin adsorbed layer of matrix, which often provides high local contrast. This was not the case with phage T2 (Pl. 3, fig. 18) however, where the result was poor. To assess whether any positive staining had occurred, these particles were removed from uranyl acetate solution by centrifugation after treatment for a few minutes. A comparison between Pl. 3, fig. 18, the negative-stained particles, Pl. 3, fig. 19,

particles treated with uranyl acetate, and Pl. 3, fig. 20, untreated particles dried down from ammonium acetate, indicates that there was no positive staining of the sheath, but that the head (presumably the phosphate groups of the DNA) had taken up uranyl ions to become much denser than untreated heads. If necessary this simple procedure could be used in other cases to detect positive staining. Results on phage ØR were also somewhat disappointing (Pl. 4, fig. 21); the apical subunits were almost completely removed and the shape of the phage rather poorly preserved. Nevertheless, it has been possible to obtain information about the structure of this phage by using uranyl acetate (Kay & Bradley, 1962).

A comparative study of a staphylococcus phage with uranyl acetate and phosphotungstate indicated the differences in appearance which are to be expected in preparations made with these materials. A phosphotungstate preparation is shown in Pl. 4, fig. 22; the head is oblong and rounded, the tail is practically structureless, but there is a knob or appendage at the tip. There is a startling difference between this appearance and Pl. 4, fig. 23 (uranyl acetate), which shows a slimmer and more sharply angled head, and a striated tail which has no appendage at the tip. A 'high and dry' head is shown in Pl. 4, fig. 24; it is dense, and doubtless stained positively, but there is a thin electron-transparent line around the dense region which probably represents the unstained protein of the head membrane. A halo of carbonaceous contamination is also visible, indicating that the particle was standing proud of the support film and hence that the three-dimensional preservation was probably good.

On the basis of these observations, results obtained with uranyl acetate should always be compared with those obtained with phosphotungstate. It is also desirable to take micrographs of particles in as many different conditions of embedding as possible. Uranyl acetate is obviously very useful though difficult to use and to interpret.

Uranyl acetate with EDTA. This mixture was tested with phages T2 and T4 under neutral conditions. For phage T2 in ammonium acetate (Pl. 4, fig. 25) it appeared unsatisfactory, though the shape of the head was well preserved. There was some improvement with phage T4 in ammonium carbonate (Pl. 4, fig. 26), preservation being good, but definition poor. These results are virtually useless; but other workers such as van Bruggen, Wiebenga & Gruber (1960) have obtained with this mixture excellent micrographs of such specimens as haemocyanin molecules. Perhaps better results might be obtained by using a different amount of EDTA at different pH values.

Uranyl nitrate at pH 5.2 with phage T2 in ammonium acetate showed the phage tail to be destroyed (Pl. 5, fig. 27), and there was no spreading. The heads were heavily stained with shape well-preserved.

Uranyl nitrate with EDTA in ammonium acetate at neutral pH value, fully embedded particles of phage T2 showed little detail (Pl. 5, fig. 28). Results with 'high and dry' particles resembled those obtained with plain uranyl nitrate (Pl. 5, fig. 29).

Lanthanum acetate. Phage ØR in ammonium acetate mixed with 1% lanthanum acetate (pH of mixture 5.2) produced high contrast but coarse background, even across holes (Pl. 5, fig. 30). It is possible that a satisfactory lanthanum acetate mixture could be developed; this ought to be of value because of the high contrast obtainable.

Thorium nitrate and chloride. These two chemicals were tested with ammonium acetate and failed for different reasons. The result with the nitrate and phage ØR shows excessive granularity (Pl. 5, fig. 31). With the chloride there were undesirable effects upon the specimen, phage T2 (Pl. 5, fig. 32), though actual negative staining and contrast was reasonable.

Effect of pH value on some negative stains

It is reasonable to suppose that the efficiency of a negative-staining material will be changed according to the pH value at which it is used. This has already been noted with phosphotungstate (Bradley, 1961*a, b*); at the same time it was found that the pH value of a mixture changed as it dried on the grid. This point will be discussed below. Different staining characteristics have also been obtained on haemocyanin molecules (van Bruggen *et al.* 1960) when using uranyl acetate + EDTA mixtures at different pH values. This matter has not been exhaustively studied here, since the best pH value to choose will depend largely upon the chemical relations between specimen and embedding material. It has been found that in general best results are obtained at near neutral pH values, the background structure of most materials being least obvious under these conditions. It is not, of course, possible to use neutral solutions of chemicals such as uranyl acetate because of precipitation of metal oxide or hydroxide, a value about pH 5.0 is satisfactory here.

Changes in pH during drying

There is little doubt that the more delicate specimens examined in the electron microscope will be damaged to a greater or lesser extent by the changes in pH value which occur in a negative-staining mixture while it is drying. These changes were measured on the most satisfactory materials as follows. Negative-staining mixture (1–2 ml.) was placed on a watch-glass and allowed to evaporate at about 50°. The percentage of water which had evaporated was measured at intervals by weighing, and the pH values determined (cold) at the same time with British Drug Houses Ltd. capillators; the accuracy is usually ± 0.1 pH unit. Towards the completion of the evaporation of the sample, the salt concentration reaches a high value; at such concentration pH measurements made with glass electrodes are of little value, and dyes are more reliable. In the present experiment, 0.005–0.01 ml. of liquid was removed from the watch-glass, mixed with an equal volume of pH capillator dye solution, and compared with the standard. The results were plotted against the percentage of solution which had evaporated. Though this procedure is analogous to the drying stage of the negative-staining process with spreading, the pH determinations took 2–3 hr., the spreading process 1 min., and the drying of sprayed droplets perhaps a few seconds.

To determine whether the presence or otherwise of phage particles had some effect on the pH curve, 10^{12} – 10^{13} particles of phage T4/ml. were added in a few cases. They had little effect on the curve, and none on the pH value reached by the mixture just before drying (see Table 1). The pH range through which a drying mixture passes depends mainly upon the relative volatility of the acids and bases constituting the salts. Results obtained with some of the unmixed solutions are shown in Table 1. When these solutions are mixed in varying proportions, curves of

almost any shape can be produced. Figure 2 shows curves for various mixtures of potassium phosphotungstate and ammonium acetate; the pH value remained virtually unchanged only when it was mixed with an equal volume of 0.025M-ammonium acetate. This particular mixture was found by trial and error and it should be possible to adjust most negative-staining mixtures in this way. Figure 2 also includes curves for uranyl acetate and sodium molybdate mixtures. Figure 3 gives curves for sodium tungstate and mixtures with ammonium acetate, and again demonstrates how changes in pH value can be controlled. This graph also shows that ammonium acetate and ammonium carbonate follow the same curve. While the pH changes noted here were relatively small (the largest was 1.6 pH units) they might affect delicate specimens and perhaps the staining efficiency to a small extent. The changes would be more important when proteins with a known isoelectric point were being studied and the pH value must be carefully controlled.

Table 1. *Changes in pH value of negative-staining solutions during drying*

Negative stain	Amount	Buffer	Amount	pH values (% evaporated)			
				0 %	50 %	75 %	100 %*
2 % PTA†, pH 7.0	1 vol.	Nil	Nil	7.0	7.0	7.2	7.8
2 % PTA, pH 4.2	1 vol.	Nil	Nil	4.2	4.0	3.8	3.4
Nil	Nil	0.1 M ammonium benzoate	1 vol.	7.2	5.9	5.7	5.6
Nil	Nil	Ditto + phage T 4	1 vol.	6.7	6.0	5.8	5.6
2 % PTA	1 vol.	0.1 M ammonium benzoate	1 vol.	6.8	6.7	6.5	5.5
2 % PTA	1 vol.	0.1 M ammonium carbonate	1 vol.	7.0	7.2	6.9	6.0
2 % sodium tungstate	1 vol.	Nil	Nil	7.3	7.9	8.2	8.8
1½ % Sodium PMA‡	1 vol.	0.1 M ammonium acetate	1 vol.	7.2	6.6	6.6	6.4
2 % Uranyl acetate	1 vol.	Nil	Nil	5.2	5.5	5.8	6.0
2 % Uranyl acetate, pH 5.2	1 vol.	0.1 M ammonium acetate pH 6.8	2 vol.	5.6	5.8	5.9	6.0
1 % Uranyl acetate, pH 4.0	1 vol.	Nil	Nil	4.0	4.3	4.4	5.0
1 % Uranyl acetate, pH 4.0	1 vol.	0.1 M ammonium acetate pH 7.2	2 vol.	4.4	4.6	4.6	5.2
1 % Uranyl acetate, + EDTA, pH 7.2	1 vol.	0.1 M ammonium acetate pH 7.2	1 vol.	7.2	6.2	6.0	6.0

* This is a theoretical value obtained by extrapolating the curves obtained by plotting pH against % evaporated, and represents the pH attained immediately before drying is complete.

† PTA = K phosphotungstate. ‡ PMA = phosphomolybdate.

The effect of negative staining on the viability of phages

The effect of different negative-staining chemicals on the viability of several phages was examined. In general there was a reasonably high survival rate when phage suspensions were mixed with the various negative-staining chemicals; uranyl

acetate was the most destructive and killed most types of phage; phosphotungstate was the least destructive. However, when phages either in negative-staining mixtures or in plain buffer solutions were dried *in vacuo*, there was no survival at all.

Size measurements

It is desirable to be able to measure the size of specimen particles in negatively-stained preparations with some degree of accuracy and reliability so that dimensions obtained can be compared with those found by techniques other than electron

Fig. 2. Curves showing pH changes in suspending media as they evaporate. ●, 2 vol. 2% PTA, pH 8.6; 1 vol. 0.1M-ammonium acetate, pH 7.2. ○, 2 vol. 2% PTA, pH 4.2; 1 vol. 0.1M-ammonium acetate, pH 7.2. ×, 1 vol. 2% PTA, pH 7.0; 1 vol. ammonium acetate, pH 7.2. △, 1 vol. 2% PTA, pH 7.2; 1 vol. 0.025M-ammonium acetate, pH 7.2. □, 2% PTA, pH 8.6. ⊙, 1 vol. 2% Na molybdate, pH 7.2; 1 vol. 0.1M-ammonium carbonate, pH 7.2. +, 1 vol. 2% uranyl acetate, pH 5.2; 1 vol. 0.1M-ammonium acetate, pH 6.8.

microscopy. With phage ØR (Kay & Bradley, 1962) different sizes were obtained by different techniques; shadowing gave the highest value and uranyl acetate the lowest. A comparison of the sizes obtained with staphylococcus phage 70 is summarized in Table 2. The discrepancies are considerable, and in the case of the head width, geometrically impossible. In examining the micrographs to determine other differences it can be seen that with phosphotungstate the head was rounded with no sharp angle; with uranyl acetate the angles were very sharp. Shadowed preparations (Pl. 2, fig. 9) have shown that heads in phosphotungstate tend to collapse and hence have a greater apparent size. But the observed discrepancy can only be

partly accounted for on this basis. The only alternative explanation is that contraction has occurred with the uranyl acetate. The following observation indicated that this was, in fact, the case. When particles of phage T2 embedded in uranyl nitrate+EDTA mixture were being examined (Pl. 5, figs. 28, 29) a difference of about 15% in head width was noted (taking into account the carbonaceous contamination on the non-embedded heads). This contraction was seen to occur in the microscope as the non-embedded particles were moved into the electron beam; it took place over a period of $\frac{1}{2}$ –1 sec.

Fig. 3. Curves showing pH changes in suspending media as they evaporate. □, 2% sodium tungstate. Δ, 2 vol. 2% sodium tungstate, pH 7.2; 1 vol. 0.1 M-ammonium acetate, pH 7.2. ●, 1 vol. 2% sodium tungstate, pH 7.2; 1 vol. 0.1 M-ammonium acetate, pH 7.2. x, 1 vol. 2% sodium tungstate, pH 7.2. 2 vols. 0.1 M-ammonium acetate, pH 7.2. ○, 0.1 M-ammonium carbonate, pH 7.2. ⊙, 0.1 M-ammonium carbonate, pH 7.2.

Table 2. *Dimensions of staphylococcus phage 70*

Negative stain	Head length	Head width	Tail length	Tail width
	(A°)			
Potassium phospho- tungstate	980	530	3000	95
Uranyl acetate	750	300	2800	85

Dimensions quoted are averages of ten measurements.

There is no doubt that the question of size measurement requires further investigation. At present the most reliable estimate must be an average of results obtained by various preparation methods. In some cases, however, the three-

dimensional preservation may be obviously good, for example in Pl. 5, fig. 33. This shows an octahedral phage head where the facets are clearly shown without any distortion. Any measurement from such a head would undoubtedly be accurate. These observations presuppose an accurate calibration of the instrumental magnification. Care must be used with polystyrene latex suspensions since the spheres are liable to appreciable shrinkage (personal observation) under electron bombardment.

Resolution

The efficiency of a technique is often judged by the resolution obtainable with it, and it is usually difficult to quote an exact figure. Brenner & Horne (1959) claimed 15 Å, but showed no pictures to support this. In the experience of the present author, it has been found that the average micrograph resolves only about 30 Å, though, on occasions when conditions are exactly right less than 15 Å can be attained. It is a fortunate coincidence that the apical subunits of phage ØR (Pl. 2, figs. 7, 10) provide a conclusive test object; in the micrographs shown they can be seen to consist of two portions, about 15 Å in size and separated by a gap of 10–15 Å. Thus, on the basis of the particle separation definition of resolution, better than 15 Å was obtained here; but it must be emphasized that this micrograph is an exception rather than the rule.

CONCLUSION

Though it is basically a very simple technique, the negative-staining method has many variations. As with replica processes, each worker appears to have his own special handling procedure, but the ultimate result is the same. Many of the real variations have been described here and they involve different combinations of materials. These often appear to give complementary information. For example, while phosphotungstate preserves fine detail better than most other reagents, the three-dimensional shapes of larger structures usually become badly distorted. These are, on the other hand, well preserved by uranyl acetate though they may have shrunk slightly. It is clear that it is dangerous to rely only on one staining material. The information given here should provide a basis for the selection of reagents for most types of specimen, whether delicate, or stable.

The author is grateful to those listed above who provided phages, and Miss M. McCulloch for technical assistance. The author would like to thank Dr D. Kay (Sir William Dunn School of Pathology, University of Oxford), Dr P. Wildy and Dr D. Watson (Institute of Virology, University of Glasgow) for valuable criticism and advice and also Professor M. M. Swann (Zoology Department, University of Edinburgh) for his help and interest.

REFERENCES

- ANDERSON, T. F. (1960). On the fine structure of the temperate bacteriophages P1, P2 and P22. *Proc. Eur. Reg. Conf. on Electron Microscopy, Delft, 1960*, 2, 1008.
BRADLEY, D. E. (1961*a*). Negative staining of bacteriophage ØR at various pH values. *Virology*, 15, 203.
BRADLEY, D. E. (1961*b*). The preparation of specimen support films. *Techniques for Electron Microscopy*, chap. 3, p. 60. Ed. D. Kay. Oxford: Blackwell.

- BRADLEY, D. E. & KAY, D. (1960). The fine structure of bacteriophages. *J. gen. Microbiol.* **23**, 553.
- BRENNER, S. & HORNE, R. W. (1959). A negative staining method for high resolution electron microscopy of viruses. *Biochim. biophys. Acta*, **34**, 103.
- BRENNER, S., STREISINGER, G., HORNE, R. W., CHAMPE, S. P., BARNETT, L., BENZER, S. & REES, M. W. (1959). Structural components of bacteriophage. *J. mol. Biol.* **1**, 281.
- DAEMS, W. TH., VAN DER POL, J. H. & COHEN, J. A. (1961). Some remarks on the morphology of bacteriophage T4B. *J. mol. Biol.* **3**, 225.
- HALL, C. E. (1955). Electron densitometry of stained virus particles. *J. biophys. Biochem. Cytol.* **1**, 1.
- HARRISON, B. D. & NIXON, H. L. (1960). Purification and electron microscopy of three soil-borne plant viruses. *Virology*, **12**, 104.
- HUXLEY, H. E. (1956). Some observations on the structure of tobacco mosaic virus. *Proc. Eur. Reg. Conf. on Electron Microscopy, Stockholm, 1956*, p. 260.
- HUXLEY, H. E. & ZUBAY, G. (1960). Electron microscope observations on the structure of microsomal particles from *Escherichia coli*. *J. mol. Biol.* **2**, 10.
- KAY, D. (1962). The nucleic acid composition of bacteriophage ØR. *J. gen. Microbiol.* **27**, 201.
- KAY, D. & BRADLEY, D. E. (1962). The structure of bacteriophage ØR. *J. gen. Microbiol.* **27**, 195.
- VAN BRUGGEN, E. F. J., WIEBENGA, E. H. & GRUBER, M. (1960). Electron micrographs of *Helix pomatia* hemocyanin. *Proc. Eur. Reg. Conf. on Electron Microscopy, Delft, 1960*, **2**, 712.

EXPLANATION OF PLATES 1-5

PLATE 1

- Figs. 1, 2. Phage T4 in ammonium benzoate and phosphotungstate, $\times 330,000$.
 Fig. 3. Phage T2 in ammonium benzoate and phosphotungstate, $\times 333,000$.
 Fig. 4. Phage T2 in ammonium carbonate and phosphotungstate, $\times 333,000$.
 Fig. 5. Phage T4 in ammonium carbonate and phosphotungstate, $\times 333,000$.
 Fig. 6. Phage 66t- in ammonium acetate and phosphotungstate, $\times 333,000$.

PLATE 2

- Figs. 7, 8. Phage ØR in ammonium acetate and phosphotungstate, $\times 333,000$, by courtesy of *Virology*.
 Fig. 9. Phage T2 in ammonium acetate and phosphotungstate, shadowed at $\tan^{-1}\frac{1}{4}$, $\times 150,000$.
 Fig. 10. Phage ØR in ammonium acetate and sodium tungstate, $\times 333,000$.
 Fig. 11. Phage T4 in ammonium carbonate and sodium phosphomolybdate, $\times 333,000$.
 Fig. 12. Phage T4 in ammonium acetate and sodium phosphomolybdate, $\times 333,000$.
 Fig. 13. Phage ØR in ammonium acetate and sodium phosphomolybdate; $\times 333,000$.

PLATE 3

- Fig. 14. Phage T4 in ammonium acetate and sodium molybdate, $\times 333,000$.
 Fig. 15. Phage ØR in ammonium acetate and sodium molybdate, $\times 333,000$.
 Fig. 16. Phage T4 in ammonium carbonate and sodium molybdate, $\times 333,000$.
 Fig. 17. Phage T2 in ammonium acetate and uranyl acetate, shadowed $\tan^{-1}\frac{1}{4}$, $\times 150,000$.
 Fig. 18. Phage T2 in ammonium acetate and uranyl acetate, $\times 333,000$.
 Fig. 19. Phage T2 treated with uranyl acetate, $\times 330,000$.
 Fig. 20. Phage T2 suspended in ammonium acetate only, $\times 333,000$.

PLATE 4

- Fig. 21. Phage ØR in ammonium acetate and uranyl acetate, $\times 333,000$.
 Fig. 22. Staphylococcus phage 70 in ammonium acetate and phosphotungstate, $\times 333,000$.
 Figs. 23, 24. Staphylococcus phage 70 in ammonium acetate and uranyl acetate, $\times 333,000$.
 Fig. 25. Phage T2 in ammonium acetate and uranyl acetate with EDTA, $\times 333,000$.
 Fig. 26. Phage T2 in ammonium carbonate and uranyl acetate with EDTA, $\times 333,000$.

PLATE 5

- Fig. 27. Phage T2 in ammonium acetate and uranyl nitrate, $\times 333,000$.
Fig. 28. Phage T2 in ammonium acetate and uranyl nitrate with EDTA, $\times 333,000$.
Fig. 29. The same 'high and dry', $\times 333,000$.
Fig. 30. Phage ØR in ammonium acetate and lanthanum acetate, $\times 333,000$.
Fig. 31. Phage ØR in ammonium acetate and thorium nitrate, $\times 333,000$.
Fig. 32. Phage T2 in ammonium acetate and thorium chloride, $\times 333,000$.
Fig. 33. Phage 1C in ammonium acetate and phosphotungstate, $\times 333,000$.

D. E. BRADLEY

(Facing p. 516)