The Use of Spraying Methods and of Volatile Suspending Media in the Preparation of Specimens for Electron Microscopy*

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Developments have been made in the use of spraying methods in the preparation of specimens for examination in the electron microscope. At the same time, two wholly volatile diluents, containing electrolytes and adjusted to normal *p*H, have been developed for use in forming the spray drops. The use of volatile electrolytes makes it unnecessary to wash the specimen after the droplet patterns have been formed on the specimen screens.

There are several qualitative and quantitative uses and advantages of the spray technique as described. The qualitative ones are these: (1) the brief drying time of the droplets helps to preserve the shapes of particles upon drying, and also allows studies to be made of rapidly reacting systems; (2) since the drop patterns are reproducibly representative samples of the suspension under investigation, qualitative assays of the particulate composition of the suspension can be made, and a comparison of the particulate composition of two closely similar suspensions can be made under ideal control conditions; (3) the droplet patterns are discretely bounded by blank areas of substrate film, and this fact makes practicable the detection of small and subtle differences between the fine structure of the specimen material and that of the substrate.

The quantitative uses of the spray technique are: (1) by use of reference particles of known numbers in a suspension it is readily possible to determine the volumes of droplets issuing from a spray-making device; (2) by use of reference particles it is also possible to make an assay of the number of other particles, such as a virus, per unit volume of mixed suspension; (3) if a suspension is highly purified, the particle weight of the material in suspension can be determined. Experiments involving the counting of about 10,000 particles show that the quantitative precision is as good as would be expected from the statistics of random sampling.

The construction and use of a spray gun are described, as well as the characteristics of two volatile diluents: ammonium acetate and ammonium carbonate.

I. INTRODUCTION

SPECIMENS for electron microscopy are commonly prepared by placing a drop of liquid containing some suspended material upon the film-covered specimen screen, and allowing the drop to dry either unassisted or hastened by the withdrawal of some of the liquid on a piece of filter paper. If the material is suspended in a non-volatile medium, such as one of the usual diluents containing salts, it is necessary to wash the microscope screen in distilled water subsequent to the initial drying of the suspension. It is generally agreed that this technique is unsatisfactory, owing to the chemical and physical changes that may occur during the drying period of several seconds to a few minutes, and to the inevitable non-uniform distribution of the suspended material as it dries on the screen. The exposure of biological materials of even moderate lability to an environment of high ionic strength, followed by immersion in distilled water, is a procedure likely to cause changes in the shapes and sizes of the specimen materials. Attempts have been made to alleviate some of these unfavorable conditions; notably by freeze-drying the specimen drop, and by treatment of the suspended material with a fixative such as formalin.

A method occasionally used since the earliest days of electron microscopy can be used to reduce many of the drawbacks of the usual procedure described above. This is a method by which the suspension is either allowed to settle on the specimen screen as tiny droplets, or is sprayed directly and forcibly upon the screen. As a result of this procedure, the screen is partially covered with discrete, roughly circular, droplet patterns from about 5 to 20 microns in diameter.

Spray methods have been described by Riedel and Ruska,[1] Haardick, Kausche and Ruska,[2] and by Cravath, Smith, Vinograd, and Wilson.[3] They appear not to have been put to any general use, however, probably because their advantages largely disappear when the specimen material is suspended in the non-volatile diluents commonly used. We have made developments in the spray technique with the primary purpose of applying it to the preparation of specimens of biological material, and have at the same time developed aqueous suspending media which are wholly volatile. The method now appears to be highly useful for both qualitative and quantitative electron microscopy. Its

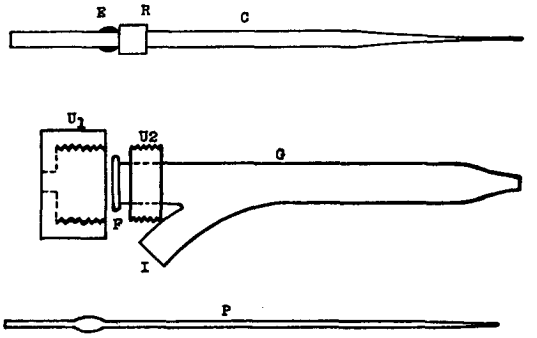

FIG. 1. Sketch of glass spray gun.

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[1] G. Riedel and H. Ruska, Kolloid-Zeits. **96**, 86 (1941).
[2] Haardick, Kausche, and Ruska, Naturwiss. **27/39**, 226 (1944).
[3] Cravath, Smith, Vinograd, and Wilson, J. App. Phys. **17**, 309 (1946).

advantages and uses will be detailed below, following a description of a spray gun and a discussion of volatile suspending media.

II. CONSTRUCTION AND USE OF A SPRAY GUN

A spray gun should be capable of depositing upon the specimen screens a pattern of discrete droplets of approximate uniformity of size, reproducibly representative of the suspension, without the presence of contaminants originating in the gun, and devoid of any sprayed material in the regions between the droplet patterns. The simple glass gun to be described has been found adequate to meet these requirements, although it is realized that other gun designs might be found to be equally adequate. Figure 1 is a schematic drawing of the gun in which the parts are shown unassembled.

The outer envelope G is made of apparatus-weight, 7 mm glass, and is provided with an air inlet I and a means for securing the insert tube C. Tube G is provided with a threaded Lucite union U_1 and U_2, with U_2 of an internal diameter less than the diameter of the enlarged flange at F. U_1 threads over U_2, but also has an unthreaded base section with a hole just large enough to fit over tube C. The tube C is provided with a rubber sleeve R and a retaining bulge at E, and upon assembly an air-tight seal is made by compressing R between the flange F and the base of U_1. The purpose of E is to ensure that the tube C will not be violently forced out of the end of G when the compressed air is admitted. Tube C is of uniform diameter except for the bulge at E and the finely drawn portion at the tip.

It has been found convenient to make a number of small pipettes P whenever a series of samples is to be sprayed. The use of these pipettes minimizes the chances of sample-to-sample contamination by restricting the area of contact between fluid and glass to the small region at the tip of C. The pipettes are inserted into C sufficiently far to cause their ends to wedge lightly into the capillary constriction in C. Each pipette is discarded after having been used with one sample of a series.

Extended trial has shown that the dimensions of the orifices are fairly critical. The I.D. of tube C should be about 0.1 mm, and the O.D. about 0.3 mm. The I.D. of the tip of tube G should be about 0.75 mm. A spraying pressure of 20 to 30 p.s.i. is satisfactory, with the gun some 15 to 20 inches from the filmed microscope screens. The screens can be mounted to a supporting surface with Scotch tape, or can be held in position magnetically. Very clean air must be used for spraying, and it appears that tank-compressed air, reduced with a suitable valve, is satisfactorily clean. The gun should be carefully aligned to cause the central region of the spray to intersect the screens; the alignment and other spray characteristics can conveniently be observed with the aid of a beam of light in a darkened room. The gun as described will deposit a convenient number of droplets from about 0.2 ml of sprayed material. A smaller orifice can be used at the tip of tube C (with higher air pressure and diminished distance from gun to screens), when the use of a smaller volume of suspension is necessary.

One of the commercially available all-glass nebulizers has been experimented with, but it has not been found to be as satisfactory for the production of representative drop patterns as is the high air-velocity gun just described. The nebulizer is not as conveniently kept free of contamination, and it does not produce drop patterns that are both discretely bounded and reproducibly representative of the specimen. We believe that the reason for the difference in the performance of the two kinds of spraying devices is that, in the case of the low air-velocity nebulizer, some material from droplets which have completely dried in transit is left in varying amounts and places upon the microscope screens.

III. VOLATILE SUSPENDING MEDIA

The preparation of materials in the form of spray-drop patterns loses most of its unique value when the suspending medium is non-volatile, since the dried salts must be rinsed off the specimen screens with consequent disturbance of the drop patterns. For many substances a satisfactory volatile medium is distilled water, but for others, particularly of a biological nature, a medium containing electrolytes is demanded. The requirements to be met by volatile electrolytes when used in electron microscopy can be stated only after consideration of the type of observation to be made. A minimum requirement would be that the chemical and physical properties of the substance, when suspended in one of the conventional diluents, should be maintained when the substance is suspended in the volatile diluent.

It is clearly necessary that the preparation of a volatile electrolyte be done in a manner calculated to avoid any chance of contamination with non-volatile material. If a non-volatile component is present as much as 1 part in 10^7 in an average spray droplet of 500 microns^3 volume, there will be 5×10^7 A^3 of residual material in the drop pattern. This amount is clearly visible in the electron microscope if it dries on the screen in a form other than a rather uniform film. Such a high degree of freedom from non-volatile contamination requires that only gaseous or freshly redistilled liquid components be used in the chemical preparation.

We have found a 2 percent solution of freshly made ammonium acetate at *p*H 7.0 to be a satisfactory suspending medium for use with several biological materials (for example: fibrinogen, serum globulins, plant viruses, and homogenized tissue suspensions). Ammonium carbonate at a similar ionic strength and *p*H has also been found to be useful. It is advantageous to consider spraying the same material suspended in both of these volatile electrolytes in turn, on account of the changes in *p*H that occur during the brief drying time

of the droplets. The ammonium acetate system will approach a *p*H of about 4.0 as it dries, while ammonium carbonate approaches a *p*H of 10. If differences of form observable in the electron microscope are believed likely to take place in a drying time of about 0.1 sec., their magnitude can be checked by spraying with each volatile system separately, or with the two systems used together in varying concentrations.

IV. USES AND ADVANTAGES OF THE SPRAY METHOD

The consequences of the use of droplets sprayed in a volatile suspending medium may be generally described thus: (1) the time taken for the specimen material to change from its free liquid environment to complete dryness is very short, (2) the droplet patterns are individually and reproducibly representative of the composition of the sprayed material, and (3) the droplet patterns are discretely bounded by areas of blank specimen film. The uses and advantages accruing from these consequences will now be described in detail.

A. Qualitative

1. *Short drying time*—The total time elapsed between the emergence of an aqueous drop of about 500 microns[3] volume from a high air-velocity spray gun and the state of complete dryness on the specimen screen is estimated to be somewhat less than 0.1 sec. The estimate is based upon the known rate of evaporation of water droplets in relatively dry air, and disregards any possible effect of the suspended material in slowing or accelerating the drying process. This drying time is to be compared with a time of several seconds to a minute or so taken by an ordinary, gross drop to dry when placed directly upon the filmed specimen screen.

The advantage of the short drying time is that the suspended material, such as a virus, for example, is exposed only very briefly to an unfavorable electrolytic and osmotic environment and has little time to be morphologically changed. There is also little time for the occurrence of gross aggregation or other particle interactions which must take place during the concentration of the solute materials during the long drying time of a large drop. A further advantage is that very small particles do not imbed themselves in the plastic film as much as they do when the film is softened and swollen by prolonged contact with a large drop of water. An important use of the fact of quick drying is in observations of interparticle reactions, where it is desirable to stop the reaction quickly and at a known instant. In this type of experiment the particulate components are mixed prior to, or during, their introduction into the gun, and the reaction is manifestly stopped within a fraction of a second after the spray drops leave the gun.

2. *Reproducibly representative fields*—It is difficult to overestimate the usefulness of a technique whereby well-defined areas of the microscope screen contain representative and complete samples of all the non-volatile material in the original suspension. This is particularly true when the suspending diluent is in itself free of non-volatile components. The common experience of microscopists is that searching for and photographing "typical" fields is time-consuming, frustrating, and is open to the dangers of subjective judgment. This difficulty has made comparisons between two samples of material, such as normal and infected biological material, relatively uncertain, with the result that the electron microscope has failed to be used to its fullest as an analytical tool. The use of drop patterns eliminates a fundamental difficulty in electron microscopy; namely, the small totally available field of search of about 0.25 mm^2. This restricted field leads to uncertainty as to whether or not the portion of the specimen of great significance lies outside the field, or is obscured by the wires of the specimen grid.

A few qualitative uses of observations on small, representative drop patterns are immediately apparent. The first use lies in making reliable assays of the particulate composition of a given suspension by photographing a few drop patterns. Assays can be made by even an inexperienced person, since the choice of what is typical is independent of his judgment. If the suspension is believed to be a fairly homogeneous one, such as a suspension of a purified plant virus, the microscope can also be used to assay sensitively the amount of particulate impurities present.[4]

The spray method allows a precise comparison to be made of the electron microscopic appearance of two different, but similarly prepared, suspensions. The comparison can be conveniently made in this way: Suspension *A* is sprayed upon a few filmed specimen screens. Suspension *B* is adjusted to have about the same concentration of suspended material as *A*, but to it is added a trace amount of an indicator substance of recognizable shape, such as tobacco mosaic virus. Suspension *B* is then sprayed upon the screens already containing the dried droplets of suspension *A*. The screens are shadowed and examined for regions where discrete, closely adjacent drop patterns of both *A* and *B* can be photographed. This technique seems to embrace all the requirements of controlled electron microscopy, since the methods of mounting the specimen materials are closely identical, even to the details of drying time and surface character of the substrate film, as well as film thickness, shadowing conditions, and circumstances of exposure in the microscope.

3. *Discretely bounded drop patterns*—A constant source of uncertainty in the examination of shadowed electron micrographs of inhomogeneous particulate material is the difficulty of distinguishing between the fine-structure of the specimen material itself and the fine-structure of the substrate film. A similar difficulty

[4] R. C. Williams and R. C. Backus, J. Am. Chem. Soc. (in press).

is present even when preshadowed replicas[5] are used, except that the source of uncertainty is not in the structure of the collodion surface but instead resides in the uncertain degree of cleanliness and discontinuities of the glass surface. When drop patterns are examined, the uncertainty largely disappears, since the patterns occupy discrete areas surrounded by areas of film whose undisturbed structure can be gauged. This separation of regions provides one with a sensitive internal control on the character and cleanliness of the surface of the substrate, and hence enhances the possibility of

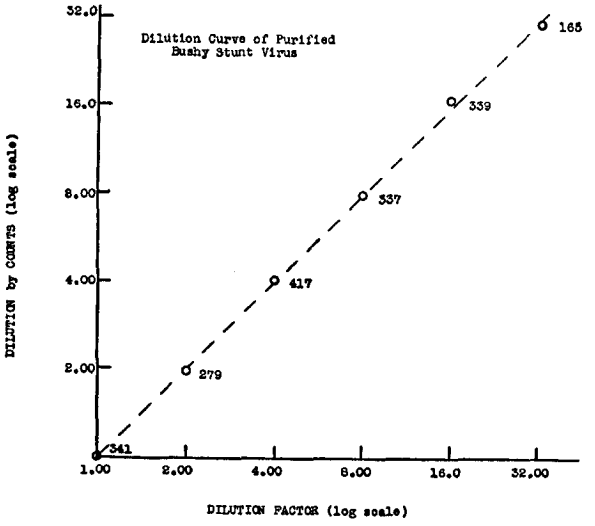

FIG. 2. Determination of the dilution of a suspension of purified bushy stunt virus by spraying and counting. Abscissae are the known dilution factors, obtained by pipetting; ordinates are the dilution factors calculated from the virus counts. The point at the origin has been arbitrarily called a relative dilution of unity. The diameter of the circles represents a 3 percent variation, while the numbers accompanying each circle are the numbers of reference particles counted in the establishment of each point.

making unequivocal observations of the presence or absence of very small particles.

B. Quantitative

The attainment of representative sample fields makes electron microscopy increasingly applicable to certain kinds of quantitative determinations. A technique to provide such fields by centrifugal deposition has been suggested by Crane.[6] A quantitative application of this technique has been made by Sharp[7] who obtained representative fields of swine influenza virus by sedimenting the particles on to a collodion surface. By counting the number of particles per unit area of the film he obtained a satisfactory correlation between counts and known dilution factors, although his results were rendered somewhat uncertain by the necessity of washing the dried buffer salts from the sedimented preparations.

[5] R. C. Williams and R. W. G. Wyckoff, J. App. Phys. **17**, 23 (1946).
[6] H. R. Crane, Rev. Sci. Inst. **15**, 253 (1944).
[7] D. G. Sharp, Proc. Soc. Exp. Biol. Med. **70**, 54 (1949).

1. *Determination of droplet volumes*—If reference particles[8] are present in a known concentration in the suspension being sprayed, a given droplet pattern will contain a number of these particles proportional to the volume of the droplet as it left the spray gun. It has been found that Dow latex particles[9] are particularly useful as reference particles, since they are obtainable in quite pure aqueous suspension, and remain intact and in uniform suspension in ammonium acetate and ammonium carbonate. The particles do not seem to interact with the biological materials we have handled, at least during the brief periods of contact necessary for their use, and their concentration in numbers per unit volume of suspension is readily determinable.[4] It is evident that one can use the latex reference particles directly in a determination of volumes of droplets as they issue from any spray-making device.

2. *Absolute virus assays*—When latex particles are mixed with some other particulate material, such as a virus, simple counts made on the droplet patterns will determine the ratio of the number of latex to the number of virus particles. If the concentration of latex is known, this ratio provides an absolute assay of the number of virus particles per unit volume of the mixed suspension. It is to be noted that this method can be used even though the suspension is not highly purified; as long as one species of particle can be recognized, it can be counted and assayed.

3. *Determination of particle weights*—The technique just described can be used to determine the particle weight of a homogeneous substance, such as a purified virus. The particle counts yield the number of particles per unit volume, and if the material is in highly purified form, this information can be combined with the value of the dry weight of material in an aliquot volume of suspension to allow the weight of an average particle to be computed. This weight in grams, multiplied by Avogadro's number, is the particle weight in molecular weight units. The advantage of this method of determining particle or macromolecular weights lies primarily in its directness, and also in the low concentration of material which can be sprayed and counted.* A satisfactory concentration is 0.0001 percent, and the total weight of material to be diluted and sprayed need be no more than 0.1 microgram.

4. *Assessment of quantitative precision*—Determinations have been made of the quantitative reliability and precision of the method of particle counts by the use of

[8] E. Chamot and C. W. Mason, *Handbook of Chemical Microscopy* (John Wiley and Sons, Inc., New York, 1938), Vol. I, p. 441.
[9] R. C. Backus and R. C. Williams, J. App. Phys. **20**, 224 (1949).
* When very dilute material consisting of small sized particles is sprayed, it is difficult to recognize the confines of the drop patterns in the electron microscope. The admixture of a small amount of highly purified tobacco mosaic virus to the suspension causes the drops to be nicely bounded by easily observed rod-like forms.

reference particles in the spray drops. Two sources of uncertainty might be anticipated: the mixing of the two kinds of particles may not be perfect, and there may be dislodgments of either species of particle from the microscope screens prior to shadowing.

The sources of uncertainty have been checked by measuring the statistical fluctuations of the counts, in a trial where particles of latex and of the bushy stunt virus were mixed in approximately equal numbers. It is found from counts of about 10,000 particles that the standard deviation of the mean ratio of particle numbers lies between 2 and 4 percent when 1000 pairs of particles are counted in 20 or more droplet patterns. Inasmuch as the statistically anticipated standard deviation of the mean ratio should be close to 3 percent, it is believed that the mixing of the particles is nearly perfect, and that they do not become randomly dislodged.

The adequacy of the entire method of quantitative assay of virus particles by electron microscopy has been checked by counts made on mixtures of reference and virus particles of greatly varying relative concentrations. Figure 2 shows the results of an experiment in which the concentration of latex particles was held constant, while the concentration of purified bushy stunt virus was varied through a dilution factor of 32. The evident agreement between the known dilution factors and the dilutions as calculated from the counts of virus and latex particles is very satisfactory, and implies that a quantitative assay of a purified, dilute virus suspension can be made as precise as the statistics of random sampling would indicate.