hydroxide, but sulfur, amine, alkali, and allethrin (or pyrethrins) must all be present for the color formation. The presence of ethanol aids the reaction. If sulfur is added as a solution in carbon tetrachloride, it is necessary to have a definite quantity present, since the addition of this solution dropwise causes a deeper and deeper color until a maximum is reached. Pure allethrolone, allethrolone semicarbazone, chrysanthemum monocarboxylic acid, and pyruvic aldehyde do not react under the conditions present. Impure allethrolone does give a red color even without sulfur. It is thought that this may be due to furan derivatives that may be formed in the cyclization process, since 2-methyl furan, furfural acetone, and furfuryl acetone give red colors without the addition of sulfur to the reaction mixture.

Under one set of conditions (without the addition of sulfur) 2 mg of natural pyrethrins gave a color reading of 35 in 12 min on the Klett-Summerson colorimeter, using the 44 filter. The same quantity of pyrethrins with sulfur present gave a reading of 277, and a like quantity of allethrin (with sulfur present), a reading of 269.

A number of substitutes for sulfur have been tried in the reaction. The addition of sodium sulfide to potassium hydroxide, ethanol, and 2-(2-aminoethylamino) ethanol gives a dull blue color that is not changed by the addition of allethrin. The addition of sulfur to this mixture causes the reaction to take place and a red-brown color to appear. Thiophene likewise cannot substitute for sulfur.

We have found the following reagent mixture satisfactory: To 25 ml 2-(2-aminoethylamino) ethanol we add 50 ml ethanolic potassium hydroxide (5 g 86% potassium hydroxide is dissolved in 100 ml ethanol and the faint cloud is filtered off through glass wool). To this mixture we add 425 ml ethanol and shake thoroughly to mix. If we add to 2 ml ethanol containing at least 2 mg allethrin or pyrethrins, 8 ml ethanol, 5 ml of the above reagent mixture and 10 mg sulfur, the color reaction occurs.

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Spraying of Particulate Suspensions Containing Infective Materials for Electron Micrographic Analysis[1]

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Applications of a representative droplet field method (*1*) for examining particulate suspensions by electron microscopy have indicated its usefulness for the absolute assay of viruses (*2*) and for comparative studies on the composition of suitable preparations (*3*). A limitation of the method has been its inapplicability to the examination of pathogenic or noxious materials because of the production of an unconfined aerosol. A method has been devised that overcomes this limitation and at the same time permits the use of micro volumes of relatively dilute solutions. The method employs a self-contained apparatus of convenient size which, following an initial evacuation, can be maintained under reduced pressure during the entire operational sequence of introduction of several samples, spraying, and removal of exposed specimen grids. The sample volume needed for producing an adequate number of droplet patterns for observations over the limited scanning area of the universal model RCA electron microscope is about 10 λ.

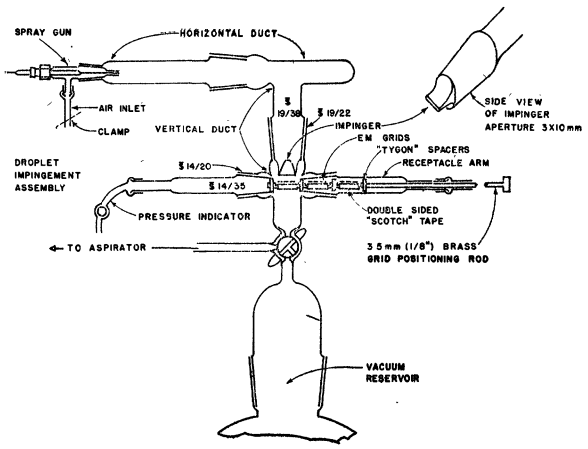

FIG. 1. Spray apparatus.

The device shown in Fig. 1 may be described in terms of three functional parts: (*a*) a 5-liter evacuated reservoir with a suitable valve for evacuation and regulation of the spraying unit, (*b*) a spray gun, and (*c*) an arrangement for droplet collection by impingement on specimen grids. In operation the apparatus is assembled and evacuated by water aspirator with the stopcock fully open. During evacuation the side arm of the spray gun remains clamped off, and the inlet end of the capillary insert is kept sealed (the sample inlet end of the capillary insert is flame-sealed during fabrication and severed to admit samples for spraying). On completion of evacuation the cock is turned through 180° and the pump connection removed. The apparatus may now be moved to a hooded area or to the vicinity of an autoclave if decontamination subsequent to spraying is contemplated.

The spray gun, shown in detail in Fig. 2, is designed to operate with the simultaneous admittance of liquid sample and air dispersant through their respective orifices into the reduced pressure area of the duct. The gun envelope orifice is made as small as practicable, since its size largely determines the rate at which the internal and external pressures equalize. In order

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[2] The author expresses his appreciation of the encouragement and counsel of Robley C. Williams of this laboratory.

to maintain as great a pressure difference throughout the sprayings as possible, entry of air through the gun is restricted by clamping the air inlet except when spraying.

An awareness of the characteristics of the spray gun is essential for securing good results. Because of unavoidable variation in dimensions of insert tips it has been found desirable to gauge the bore of each insert by examination with a calibrated × 20 microscope as they are prepared. Although tips of finer bore than that indicated in Fig. 2 may be used, greater time is required for spraying to obtain adequate numbers of droplets, with a consequent dissipation of pressure differential. The rate of feed of sample material through the capillary while spraying should be about 1 λ/sec. A control preparation of indicator particles in the solvent used for the stock suspending medium should be sprayed. If this solvent control does not result in the production of "clean" droplets, a contamination of the droplets during formation, in flight, or on contact with the substrate may also be suspected.

Although provision is shown in the impingement assembly for collections of droplets on three sets of grids, six or more consecutive sprayings and collections may be done on one evacuation when using a 5-liter reservoir. The sets of electron microscope grids

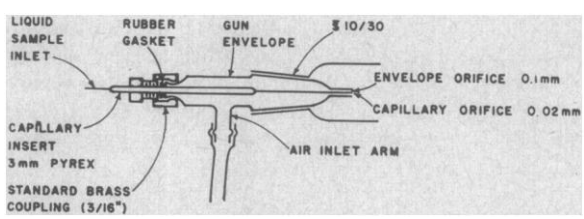

FIG. 2. Spray gun enlarged.

are carried in flattened recesses in the positioning rod between protective spacers cut from Tygon tubing as shown in the impingement assembly in Fig. 1. Scotch double-coated tape is used for holding the grids securely to the rod. After exposure to the spray, the grids are transferred for shadowing or for direct observation, with due regard for the infectiousness of the agents sprayed. The closely fitted spacers also serve to limit droplet impingement to those grids properly positioned across the duct. The impinger is constricted to an approximately 3 × 10 mm rectangular aperture to maximize the opportunity for droplets to strike the grids.

After spraying, the grids are removed in the following manner. The grids are retracted into the arm in which the positioning rod is mounted. With the gun air inlet open the stopcock is closed until the pressure indicator begins to inflate but remains partially collapsed, ensuring the continuance of a small negative pressure within the duct. The receptacle arm is disjointed from the assembly, and its contaminated end inserted into a protective tube. The opening in the duct resulting from removal of the arm is stoppered. If desirable, the gun may also be withdrawn and kept

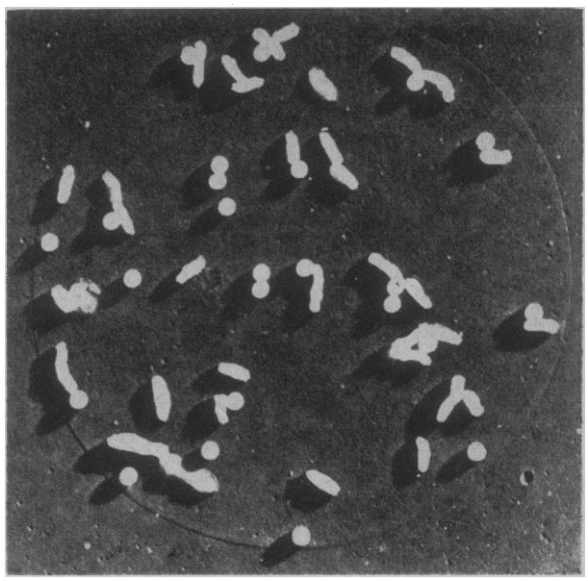

FIG. 3. Droplet pattern.

intact while the rest of the apparatus is being decontaminated. Decontamination following the spraying of pathogens can be done by autoclaving or by inspirating an effective volatile disinfectant such as chlorine. A port in the apparatus should, of course, be cotton-plugged to admit steam when autoclaving.

The relationship of the lengths of the horizontal and vertical sections of the duct determines in part the ratio of the area of the droplet pattern in the microscope to the volume of the droplet as formed at the gun orifice, since the droplets are evaporating solvent en route to the grids. A relatively long horizontal section will permit the drying of larger droplets to a size that can be conveyed around the 90° bend into the vertical section. The length of the vertical section, including the impinger element, determines the extent to which the small droplets will be further reduced in size before impinging on the grids. If the vertical duct length is too great, permitting the complete drying of many droplets to their residues, the yield of droplet patterns will be too low for convenient observations to be made. The nonvolatile material left on complete drying of droplets in flight is seldom seen on the microscope grids, indicating that it is swept away from the grids by the air stream. Lengths of approximately 10 cm for both ducts have been found to give good results.

A droplet pattern of a suspension containing the particles associated with Q-fever—*Coxiella burnetii* (Derrick), Lederle antigen—mixed with polystyrene latex (PSL) (*1*) indicator particles is shown in Fig. 3. The number of PSL spheres, sprayed in a concentration of 7×10^9/ml with the *Coxiella*, indicates the volume of the droplet as formed at the gun orifice. The concentration of the organism may be calculated from the simple relationship:

$$\frac{\text{Organism count}}{\text{PSL count}} \times \text{PSL/ml} = \text{organisms/ml.}$$

Counts on a number of droplets must be made and the data examined statistically in order to establish the reliability of the techniques used in specimen preparation for each material studied. Individual patterns suitable for counting represent volumes of the order of 10^{-8} ml of original suspension. Since it is desirable for assay purposes to have at least 10 organism particles in each droplet pattern, it is apparent that an approximate minimum concentration of 10^9 organisms/ml is required. Inasmuch as a volume of only 10 λ is required for spraying, however, a total of 10^7 particles is sufficient for making a numerical assay.

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Schwarzschild-Villiger Effect in Microspectrophotometry

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Since Caspersson's pioneering work (*1*) in microspectrophotometry, this technique has yielded, especially in recent years, extensive applications in several modified forms (*2–4*), and has revealed many interesting problems in various fields of quantitative cytochemistry. The technique hitherto reported involves, however, a serious defect, which sometimes causes an error of large magnitude in the measured value of the light transmittance of a minute part of a cell nucleus, etc., thus leading to erroneous conclusions concerning the amount of its contents.

The defect in the usual measuring methods is that they are not free from the Schwarzschild-Villiger effect (*5, 6*). This effect, found by Schwarzschild and Villiger in 1906 in the microdensitometry of photographic plates, means that, in the measurement of the transmittance of a minute part of a photographic plate, the measured value would be unduly and sometimes seriously enhanced whenever the illuminating light flux is not limited to the part on the plate. The origin of the effect is readily conceivable. When the illuminating flux is spread over a wide area, including the minute part in question, the light passing through the surrounding portion may cause flare light, owing mainly to the internal reflections and scattering of the light in the image-forming optical system; and the flare light, being added to the image of the part in question, enhances the value of the measured transmittance. Thus the effect is especially prevalent when the transmittance of the part is low and that of its surroundings high, as in the case of the microdensitometry of the photographic image of a star or a spectral line. The circumstances are the same in microspectrophotometry, and the effect is serious in the case of a deeply stained cell nucleus embedded in a transparent background.

The main part of the flare light is caused by the internal reflections of light at each air-to-glass surface of the photomicrographic system, and the effect can readily be estimated quantitatively. If a light flux of one unit enters the lens system composed of m air-to-glass surfaces, all having the same average reflectance $\bar{r}$, the part $(1-\bar{r})/\{1+(m-1)\bar{r}\}$ of the flux is transmitted, of which the part $(1-\bar{r})^m$ is utilized for imagery, the effect of light scattering and absorption being neglected. Thus the ratio θ of the flare flux to the total transmitted flux is given by

$$\theta = \left\{ \frac{1-\bar{r}}{1+(m-1)\bar{r}} - (1-\bar{r})^m \right\} \Big/ \frac{1-\bar{r}}{1+(m-1)\bar{r}},$$

which is the same as the ratio of intensity of illumination of the flare light (due to internal reflections only) on the image plane to the actual intensity of illumination on the same plane, provided the brightness of the object is uniform and its dimensions are sufficiently large—i.e., comparable with the effective focal length of the image-forming system. The values of θ for various values of m are shown in Table 1, for $\bar{r} = 0.05$

TABLE 1

m	θ (%) for $\bar{r} = 0.05$	θ (%) for $\bar{r} = 0.01$
1	0.00	0.00
2	0.25	.01
3	0.72	.03
4	1.40	.06
5	2.26	.10
6	3.27	.14
7	4.44	.20
8	5.73	.27
9	7.12	.35
10	8.62	.43
11	10.20	.52
12	11.84	.62
13	13.54	.72
14	15.29	.84
15	17.09	0.97

(for all usual optical systems) and $\bar{r} = 0.01$ (for coated optical systems).

If the number m of the photomicrographic system from the specimens to the image plane is 13, as is the case in our system—namely, 7 in an oil-immersed objective (f = 1.8~2 mm), 4 in a Huygenian eyepiece, and 2 in a reflection prism—and if the specimen is illuminated by a beam of light several millimeters wide, then the flare light caused by internal reflections only amounts to *ca* 13.5%, and the transmittance of a deeply stained nucleus having the true transmittance

[1] The author wishes to extend his sincere thanks again to Z. Koana, of our department, who has demonstrated the influence of the Schwarzschild-Villiger effect in microspectrophotometry and guided him throughout the photometric work, and to A. Sibatani, of the Institute for Microbial Diseases, University of Osaka, for the preparation of the microscopic specimens.