Applications of Metallic Shadow-Casting to Microscopy\textsuperscript{1,2}

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The factors which determine image contrast in optical and electron micrographs are discussed in relation to a new metal shadow-casting technique whereby the contrast of images is greatly increased by depositing obliquely a thin film of metal on the microscope preparations. Further advantages of the use of shadow-casting are described, and an estimate is made of the lower limit of size of objects which should be observable by shadow-casting. Examples are given and illustrations are shown of the applications of this technique to the electron microscopy of particles of macromolecular dimensions, of replicas of such particles, and of surface replicas prepared in several ways.

\section*{INTRODUCTION}

A GOOD micrograph gives clear and accurate information about the size, shape, and structure of the object from which it is derived. Whether one is dealing with microscopy involving light or electrons, the limit to what can be seen in a micrograph is in the end determined by the resolving power of the lens system and by the contrast between adjacent elementary regions of the object. Most of the contrast in the optical microscope results from differences in absorption of light within the preparation. Many important classes of objects, particularly those of biological origin, have in themselves little variation in absorption for visible light, but this restriction on microscopic examination, of course, has been in large measure eliminated through the development of elaborate methods of differential staining using dye-stuffs and other coloring agents, and to a lesser degree through the use of ultraviolet light. Under these circumstances the magnification, and the smallness of detail that can be observed with the optical microscope, are set by the attainable resolving power, i.e., by the wave-length of the light used.

Conditions are somewhat different in electron micrography at the present stage of its development. Contrast in an object illuminated by a beam of electrons is not due to differential absorption but rather to an unequal amount of scattering of electrons at different points in the preparation. The visibility of details of an object resulting from the contrast they introduce into an electron micrograph is thus determined largely by the amounts of matter contained in these details. Such amounts of matter may be so small that, especially for objects resting on a supporting membrane, insufficient contrast rather than inadequate resolving power of the microscope fixes the lower limit of visibility. The present paper is devoted to a discussion of some of these limitations arising from insufficient contrast, and to a description of a technique by which the contrast in specimens for microscopy can be greatly enhanced.

The image responsible for an electron micrograph is formed by focusing the electrons that penetrate the specimen and its substrate. The contrast in the final image is a result of failure of the lenses to focus accurately those electrons scattered through relatively large angles while passing through specimen and substrate. If these widely scattered electrons are allowed to strike the photographic plate they cause a generally distributed fogging. Maximum of contrast is therefore achieved by employing a small objective aperture which will exclude such widely deflected electrons and pass only those that can be focused with precision by the objective and projector lenses. All the electron micrographs described in this paper have been made with an RCA Type EM6 instrument equipped with an objective aperture.

Simple considerations make evident the lower limit of size of biological and other objects of low density which can be recorded by direct photography with the electron microscope. One can, for example, scarcely expect to observe

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Fig. 1. An aluminized collodion replica of a machined brass surface. 10X

directly the details of surface structure on a bacterium. Such an organism will have a thickness of not less than several tenths of a micron and it will be supported on a film of collodion or Formvar\(^4\) which supplies additional scattering matter. It is evident that surface details which provide variations in thickness of amounts less than, say, 0.02 micron will introduce totally imperceptible differences in scattering and contrast in micrographs. In the range of particles smaller than bacteria it is clear that none can be detected if its scattering is insignificant compared with that of the substrate on which it rests. All organic substances have about the same low scattering for electrons, and experience shows that under the most favorable conditions it is hard to detect the existence of particles supported by a substrate and having diameters less than about 0.01 micron. By the same reasoning it is impossible to form an accurate idea of the shapes of much bigger particles. Unless they are flat or are enclosed in thick membranes, the outlines of even relatively large organic objects must be diffuse on direct electron micrography, since at some point within their geometrical edge they will have thinned down to such an extent that the scattering is no longer considerable compared with that of the substrate.

The same kind of diffuseness of fine detail is observed in an electron micrograph of a collodion or Formvar\(^4\) replica of an etched metal or other rough surface, and it has usually been concluded\(^5\) from this that such replicas are not sharp. The work described in this paper shows that such simple replicas are faithful to the surface from which they are stripped and that their apparent lack of sharpness arises from the insufficient contrast introduced by their small scale variations in thickness and scattering. This conclusion has great practical importance because replicas made with polymers in solution are especially convenient to prepare, and can be stripped from many surfaces and materials that cannot be subjected to the high temperatures and pressures involved in making polystyrene\(^5\) and other molded replicas.

**THE SHADOW-CASTING TECHNIQUE**

In a previous paper\(^6\) we have described a technique for enhancing contrast in electron microscopic preparations which we have called shadow-casting. Briefly it consists in coating the specimen obliquely with a very thin film of metal prior to the microscopic examination. The coating is accomplished in an auxiliary vacuum chamber by means of a filament containing the metal to be deposited. The effect of the oblique deposition is to cause the higher elevations in the specimen to cast total or partial metallic "shadows" on the sides or slopes away from the filament. When the shadow-cast specimen is photographed, it yields a negative that shows light and dark areas whose degree of darkness, as a measure of the thickness of deposited metal, is a function of the angle between the tangent planes of the areas and the direction to the filament during evaporation. Such a negative, or negative print, of a shadow-cast specimen gives the same impression that is produced by a picture taken with light reflected from an obliquely illuminated landscape.

Any substance that does not exhibit structure of its own in thin films and does not migrate after deposition on the specimen could be used for shadow-casting, but it is clear that only the

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\(^3\) Formvar 15-95 is a polyvinyl formal supplied by the Shawinigan Products Corporation, New York, New York.


heavier elements can provide the extremely thin films of great scattering power that are needed in electron microscopy. Very thin films are generally desirable since they cause a minimum of alteration in the geometrical outline of the specimen on which they are deposited. Chromium yields a notably structure-free film and is ideal for shadowing relatively large objects such as bacteria, but the considerable thickness of the requisite film (ca. 70 Å) makes it unsuitable for delineating objects of macromolecular dimensions. For such smaller objects gold in a computed thickness of ca. 8 Å on the plane of the substrate gives satisfactory results.

Shadow-casting has a number of desirable consequences, first of which is that the heights or thicknesses of discrete objects that are not wholly flat are readily estimated from the lengths of the shadows they have cast. Relative heights can of course be obtained in other ways, for instance by stereoscopy, but these other procedures are more laborious, and with them it is hard to be sure which areas are high and which are low. Shadow-casting brings out in great relief the surface contours of the specimen by enhancing the scattering power, or optical effectiveness, over the entire preparation and by causing this increase to vary with the contours and to concentrate in the surface layers of the specimen. The type of detail brought out by shadowing can be varied by altering the thickness of the evaporated layer; for example if it is so thick that its optical effectiveness exceeds that of the rest of the object, it will portray almost exclusively the details of the surface. Shadowing also affords contrast between areas whose elevations in the specimen are similar but whose slopes are different, or between areas whose slopes are the same but are separated by regions of different slopes. Furthermore it makes clearly evident objects so small or thin that by themselves they would not produce a minimum contrast against the background of the rest of the specimen. Experience indicates that an ob-

**FIG. 2.** An aluminized “Faxfilm” replica from an anodically oxidized high speed drill. 500X.

ject even smaller than the lower limit of resolution of the electron microscope as it now exists can be made to cast a partial or total shadow at some highly oblique angle of illumination and that it can be detected by the existence of this shadow.

**SHADOW-CASTING FOR OPTICAL MICROSCOPY**

Shadow-casting will increase the contrast on replicas which are to be studied and photographed with the optical microscope, as well as with the electron microscope. Lower magnifications will commonly be employed for examination with visible light, and the metallic film that produces the shadows need not be unusually fine grained. As a consequence of this freedom of choice of film, aluminum of such a thickness that it is partially opaque to visible light can be used as a convenient shadowing metal for such “visual” replicas. We have made satisfactory visual replicas starting with dissolved collodion or Formvar, or with the commercial replica film process known as “Faxfilm.”

Figure 1 is a photograph of an aluminum-shadowed collodion replica of a machined brass surface, magnified ten times. Figure 2 shows an aluminized “Faxfilm” replica of an anodically oxidized surface of a high speed drill, magnified 500 times. Shadowing brings out much fine detail in this “Faxfilm” replica that could not be seen otherwise. Figure 3 is an aluminized collodion replica.

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11. Faxfilm, R. D. McDill, Cleveland, Ohio.
of a machined steel surface at a magnification of 1000 times. The contrast in such shadowed objects is so high that the condenser of the microscope can be employed at the full aperture necessary for maximum resolution.

**SHADOW-CASTING FOR ELECTRON MICROSCOPY**

1. Replicas of Surfaces

The electron microscope has proved to be especially useful in examining replicas of surfaces of many types of objects. Several methods of preparing these replicas have been described.\(^\text{13}\) Two of them, however, are in greatest current use: the Formvar process, as developed by Schaefer and Harker, and the polystyrene-silica method of Heidenreich and Peck.

As stated above, a Formvar, or a collodion, stripping lacks sharpness when viewed at high magnification in the electron microscope, and this generally has been ascribed to its intrinsic lack of sharpness of outline and faithfulness to the surface from which it is drawn. We have found on the contrary that replicas of this sort appear geometrically sharp after being shadowed, and that their unsharpness when photographed before metal deposition must be ascribed to a lack of contrast between regions of nearly the same thickness.

\(^{13}\) H. Mahl, Zeits. f. tech. Physik 21, 17 (1940); *ibid.* 22, 33 (1941); Metalwirtschaft 19, 1082 (1940); V. K. Zworykin and E. G. Ramberg, J. App. Phys. 12, 692 (1941); V. J. Schaefer and D. Harker, see reference 4; R. D. Heidenreich and V. G. Peck, see reference 5.

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**Fig. 3.** An aluminized collodion replica of a machined steel surface. 1000X.

**Fig. 4.** An all-metal chromium-shadowed replica of a glass diffraction grating.

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\(^{13}\) R. D. Heidenreich, see reference 5.
deposited silica film, introduces no contrast in the micrographs made of it. In this case the metal, which does not migrate after deposition, can be expected to form a film varying in thickness over the contours of the surface according to the cosine law, thereby producing thinner films on highly tilted areas as measured normal to the area. As a result the effective thickness of the film as seen by the electron beam will be sensibly constant over the entire replica and the result will be an almost complete lack of contrast.

We have found a number of different procedures satisfactory for preparing replicas, depending on the surface to be reproduced and the use to which the replica and its photograph are to be put. At one stage or another, however, we invariably include a high vacuum deposition of a thin metallic film at a known oblique angle.

a. All-metal replicas can be made of glass and of many crystalline surfaces. One way to make them involves evaporation from three filamented. First there is deposited upon the original surface, at normal incidence, an extremely thin (ca. 20A) film of acid-, or water-, soluble material such as copper or NaCl. This is followed by a film of chromium 100A thick evaporated also at normal incidence to provide body to the replica. The third film, which introduces contrast, can be another 100A of chromium deposited obliquely.

![Fig. 5. A gold-shadowed collodion replica of the diffraction grating of Fig. 4.](image)

![Fig. 6. A chromium-shadowed collodion replica of a surface of a used roller bearing.](image)

The specimen with its attached films is then immersed in dilute acid or in water in order to float off the coherent film of chromium, and this is then picked up on a microscope screen by "fishing." Figure 4 shows such an all-metal replica of a 15,000 line-per-inch glass diffraction grating.¹⁴ The intermediate soluble layer has in our experience introduced minute pits that make this type of replica unsuitable for magnifications exceeding ca. 20,000 times. The all-metal replica is chiefly useful when, as for calibration of magnification, one wants a specimen that can be relied on not to expand or shrink perceptibly while being made, and that does not charge up and distort under the impact of the electron beam.

b. Shadow-cast collodion replicas¹⁵ are especially easy to make and to use, and with a fast evaporating unit the time elapsed in passing

¹⁴ All the electron micrographs of this paper are from negative prints of intermediate contact positives on lantern slide emulsion. The scale of magnification in each instance is indicated by the one micron scale underneath the photograph.

from the original specimen to the finished replica can be as short as 15 minutes. The average time required to make such a replica, or a corresponding one with Formvar in place of collodion, is further reduced since a great many strippings can be shadowed at one time. For the sake of comparison between the all-metal and the collodion-base processes, there is reproduced in Fig. 5 a collodion-gold (10A deposited at a four-to-one angle) replica of the same glass diffraction grating employed to make Fig. 4.

Figures 6 to 11 are additional examples of electron micrographs of replicas prepared by this collodion-metal process. All the collodion strippings for these replicas have been shadow-cast at angles of five-to-one (arctan 1/5) either with a film of chromium ca. 80A thick or with ca. 8A of gold.

Figure 6 is a chromium-shadowed replica of the inner surface of the outer cone of a used roller bearing. This picture illustrates a great advantage of the process: it can be employed with many kinds of intact objects whose shapes, sizes, and locations are such that molded replicas could not be drawn from them.

Figure 7 is a replica of an accidental scratch on a glass microscope slide. It shows the unusual smoothness of the undamaged glass surface as well as the large amount of small scale tearing and flowing in the scratch.

Figure 8 is a gold-shadowed replica of the surface of a diamond polished parallel to a (110) plane. The approximately circular areas about one micron across correspond to elevations on the polished surface, and in this field a sharp minute pit can be seen near the center of each elevation. The residual polish marks are clearly seen running parallel across the surface of the diamond; the depth of the smallest of these is ca. 50A.

Figures 9–11 are gold-shadowed collodion replicas of the polished and etched surfaces of pieces of steel, of 60-40 brass and of magnesium. They illustrate the ease with which determinations can be made of relative heights and depths in such specimens. This is particularly evident in Fig. 11 where four distinct levels can be seen on the surface of the replica. The center is a high triangular peak surrounded by a slightly raised region of roughly triangular shape; around this
is the general level of the replica surface which is partially covered with nearly circular pits.

c. Replicas can also be made by depositing first a metal film obliquely on the surface of the specimen. If the film is sufficiently thick and the specimen has an appropriate surface it can sometimes be stripped directly to yield a simple all-metal replica. Otherwise the film can often be backed up by forming on it a supporting collodion film and stripping the two together from the underlying specimen. This process does not appear to be applicable to most etched metallic surfaces but, as will be indicated at a later point, it provides a very valuable way of studying macromolecules and other minute particles that can be deposited on a glass surface prior to being shadowed.

2. Specimens mounted on Substrates

The advantages of shadow-casting discussed earlier in this paper are all applicable to electron microscopic preparations in which the object of study is mounted for examination on a collodion or Formvar substrate. Figures 12 to 14, and the photograph on the cover of this Journal, have been chosen to illustrate the applications that
have been made of shadow-casting to the observation of biological objects of very different sizes when mounted on collodion.

Bacteria can conveniently be used to demonstrate the kind of new information that can be brought out by shadow-casting relatively large objects. Figure 12 is a photograph of chromium-shadowed typhoid bacilli which have been given an exposure in the electron microscope just sufficient to show properly the collodion background and such small details on it as the bacterial flagella, both intact and fragmented. Though the organisms themselves are underexposed it is nevertheless possible to see something of their surface structure. Figure 13 contains a group of old cells from a culture of *Staphylococcus aureus*. They have been shadowed with chromium and exposed in the electron microscope long enough to bring out much surface detail that is not otherwise seen. The way in which shadow-casting supplements the direct electron micrography of large biological objects such as bacteria is evident from these pictures and others that have been published. Since it concentrates scattering in the superficial layers of the object, it has necessarily

the general effect of hiding any inner structural details that may exist. Hence, for the most complete photographic survey of such objects three kinds of photographs should be made: micrographs of the unshadowed preparation, brief exposures, and prolonged exposures of the preparation after shadowing. In this way one obtains information regarding inner structure, structure around the periphery and adjacent to the object, and details of its surface.

In photographs we have published elsewhere of influenza virus particles\(^a\) and of tobacco mosaic virus fibrils\(^b\),\(^c\) we have demonstrated the application of the shadow technique to biological entities too small to be seen by optical microscopy. We have also shown that the technique permits the unequivocal photography of the elementary molecular particles of such macromolecular substances as the hemocyanins.\(^d\) Figure 14 is a gold-shadowed preparation (at ten-to-one angle) of a very dilute ultracentrifugally purified solution of the hemocyanin from the horseshoe crab, dried on a collodion sub-

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strate. A few molecules distributed singly and in small clusters can be seen lying on the sharply granular collodion background. These molecules, while clearly visible and casting well-defined shadows, are not much bigger than the individual collodion granules; and it would be hard to distinguish much smaller particles from the collodion on which they rest. In other words, the fine structure of the supporting collodion membrane itself sets a lower limit to the size of particles which can be mounted on it and investigated.

The picture on the cover of this Journal is another example of the photography of macromolecules supported on a collodion membrane and shadowed with gold (deposited at a five-to-one angle). The bushy stunt virus, whose elementary particles are the small spherical bodies covering most of the field of the micrograph, is known to crystallize readily from purified solution, and this tendency of the molecules to form regular arrays is very apparent in this figure. Other photographs demonstrate how similarly oriented molecular layers pile one on top of another to produce the three-dimensional symmetrical arrangements of molecules that constitute a true crystal and are responsible for its distinctive properties.

3. Preshadowed Replicas

It is evident from a photograph such as Fig. 14 that, in order to investigate molecules the size of the granules seen in a collodion film, the molecules should be on a smoother substrate when shadowed. We have found that one way to do this is to employ a technique which is essentially that mentioned above under 1c. A drop of the solution to be studied is first placed on a piece of clean, polished glass and allowed to evaporate to dryness. The glass with the desiccated material adhering to it is next shadowed obliquely with ca. 8A of gold. Since such a thin film is too fragile to be handled directly, it is backed by the usual thin film of collodion or Formvar. The polymer and the metal films are then stripped together from the glass and mounted on a specimen screen. Figure 15 shows a micrograph

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of a preshadowed replica of limulus hemocyanin molecules for comparison with Fig. 14. The molecules themselves appear equally well defined and of the same size on both photographs, but since the collodion was not metal-shadowed in the replica preparation for Fig. 15, its granular fine structure is not brought out. Obviously, far smaller particles can be distinguished and photographed on the smooth background of Fig. 15 than on a pebbly one such as that of Fig. 14.

Examination of a photograph of this type immediately raises the question of whether or not the particles that are being studied are removed with the stripped films. When dealing with objects as large as bacteria we have found that they sometimes are retained in the replica and sometimes left on the glass; which is the case in any particular instance is obvious from the opacity of the image in the electron microscope. With very small particles like the molecules of hemocyanin it makes no practical difference which is true because the electron scattering power of the metal film greatly exceeds that of the particles themselves.

Preshadowing also makes it possible to study the intimate structure of collodion and of other highly polymerized substances which must be dissolved in organic solvents that would attack the materials ordinarily used as substrates. This application is illustrated in Figs. 16 and 17. Figure 16 shows the nitrated cellulose fragments that make up collodion, as photographed in this way. The object for the left half of Fig. 16 was collodion very greatly diluted with amyl acetate and spread on glass to dry. The molecular fragments are thread-like aggregates, of various lengths, of the same granules that cover the entire surface of Fig. 14. For the right half of Fig. 16 the collodion solution was not quite so dilute. It shows how, in the formation of a continuous film, these beaded threads pack together with their long axes parallel. At the top of this figure the cellulose threads are widely spaced; towards the bottom the film is nearly continuous.

A preshadowed gold replica (at a ten-to-one angle) of polystyrene dissolved in ethyl bromide has been photographed to yield Fig. 17. The fragments strewn over the glass background vary much more in size than do those seen in the collodion photographs and some of them are of an
exceedingly small cross section. Measurements of shadow lengths indicate that some are not more than 15Å high. Though it may not be too apparent in the print, inspection of the original negatives shows that the large droplets on this and other photographs are aggregates of the smaller fragments such as those of Fig. 17. It is clear from our experience with many pre-shadowed replicas that the minimum of particle size which can be detected on them is now set by the smoothness and cleanliness of the glass surface employed. At least for the very small areas needed for this type of work, glass can be found and cleaned so as to be free from abrupt variations of surface elevation within an amount not exceeding five to 10Å, as judged by observed shadow lengths, and hence it can be concluded that electron microscopy makes it feasible to detect the existence of sharply defined objects of this range of height.

The Electrical Resistance of Iron Wires and Permalloy Strips at Radiofrequencies

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The ratio of a.c. to d.c. resistances of iron wires and permalloy strips has been measured in the frequency range 1.5–6.0 megacycles per sec. Empirical equations obtained are compared with existing theoretical equations derived on the assumption of constant permeability.

I. INTRODUCTION

WHEN an electric current flows through a cylindrical conductor in a direction parallel to the axis of the cylinder, magnetic flux lines are set up in circular paths concentric with the axis. If the current is alternating, the time variations of this magnetic flux induce voltages in closed paths in the radial planes of the cylinder. The direction of these induced voltages is such as to decrease the longitudinal current in cylindrical elements of small radii and increase it in elements of larger radii. Thus eddy currents cause the current density to be a maximum at the surface and to decrease as the distance from the surface is increased. As a result of the concentration of current near the surface of the conductor, the a.c. resistance of the conductor is greater than the d.c. resistance. The phenomena here described are usually referred to as skin effect. Since the induced voltages in the radial planes are proportional to the time-rate of change of magnetic flux and the eddy currents produced by these voltages are proportional to the conductivity of the conductor, skin effect phenomena become more pronounced with increase in frequency, magnetic permeability, and electrical conductivity. An increase in any or all of these factors results in a decrease in the depth of penetration of the current into the conductor and an increase in the ratio of a.c. to d.c. resistance. This statement applies also to conductors of rectangular sections or of other configurations.

Exact formulas for the a.c. to d.c. resistance ratio for straight conductors of circular cross section have been developed by Maxwell, Heaviside, Kelvin, and Rayleigh. By the application of Maxwell's field equations and Ohm's law, this ratio is expressed in terms of a zero-order Bessel function of an argument involving the square root of the product of frequency, permeability, and conductivity.¹ For values of this argument of ten or greater, the solution reduces to the approximate formula

\[ R/R_0 = 0.25 + 1.57d (f \mu \sigma / 10^9)^{1/2} \]

in which \( d \) is the diameter of the wire in cm; \( \mu \) the