THE MEASUREMENT OF MASS, THICKNESS, AND DENSITY IN THE ELECTRON MICROSCOPE

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ABSTRACT

A description is given of quantitative methods using the electron microscope which can be applied to specimens with much smaller dimensions than those which can be used with the established cytochemical methods based on the use of the interference microscope and the techniques of ultraviolet and x-ray absorption. A discussion of electron scattering shows that under chosen operating conditions in the electron microscope the effective total mass-scattering coefficient S of a specimen is almost independent of its chemical composition. An order-of-magnitude agreement is observed at four accelerating voltages between experimental total scattering cross-sections for polystyrene and theoretical values for carbon. The contrast in a micrograph taken under standardised conditions is interpreted in terms of differences in specimen mass-thickness. The measurement of mass, thickness, and density of discrete particles and thin sections in the absence of sublimation is discussed in terms of relevant object models on the assumption of a constant, experimentally determined, value of S. The validity of the proposed methods was examined by measuring the masses of the heads of ram spermatozoa (about 7×10^{-12} gm.) and T2 bacteriophage (about 3×10^{-16} gm.) in the electron microscope. The values agreed reasonably well with those found by interference microscopy and sedimentation-diffusion measurements, respectively. Errors in S and magnification due to contamination and their effects on the results are considered in detail. An application of the methods to a typical electron microscope specimen was demonstrated by measuring the mass of heads of the T2 bacteriophage after staining with uranyl acetate. Errors of measurement are discussed and a minimal measurable mass estimated. Further applications of quantitative electron microscopy are proposed.

INTRODUCTION

Quantitative cytochemical measurements of mass, density, and thickness by the methods of interference microscopy, absorption of x-rays, and absorption of ultraviolet light are well known (see review articles by *e.g.* Davies (8), Engström and Lindström (11), and Walker (33), respectively). The lower limit of mass measurement in interference microscopy, which we may take as representative of the above methods, is about 10^{-13} gm. and there is an obvious need for a method of mass measurement which is valid for the smaller biological organisms observable in the electron microscope (*e.g.* viruses, bacteriophages, bacteria) and for density and thickness measurement on small areas in thin sections.

Marton and Schiff (24) first described a method of measuring the thickness of isolated specimens in the electron microscope and implicit in their analysis is the possibility of the measurement of the mass and density of such specimens. Hall

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(17) measured the relative increase in mass-thickness (*i.e.* mass per unit area in the plane of the specimen) of two types of virus after the application of electron stains, and, using a predetermined calibration of his electron microscope, was able to make estimates of the virus density. Relative measurements similar to those of Hall have recently been made by Amelunxen (1). The theory of quantitative electron microscopy has been discussed by Krüger-Thiemer (20) and Zeitler and Bahr (38) and following the latter paper Bahr (2), using theoretical contrast parameters which he had shown to be in reasonable agreement with experiment for the conditions of measurement, determined the mass of microsomal particles to within an estimated error of about 30 per cent. Measurements of the mass-thickness of disrupted cell membranes, again using theoretical electron-scattering cross-sections, have been made by De and Sadhukhan (9). Quantitative aspects of electron staining have been surveyed in a recent paper by Cosslett (6).

Both the earlier discussion of Zeitler and Bahr and their most recent paper (39) are based upon the elastic electron-scattering theory of Leisegang (21) and so also are those measurements mentioned above which have used theoretical contrast parameters. In the light of recent theoretical and experimental work (to be mentioned later) it seems clear that inelastic scattering is as important as elastic for elements of low atomic number and for the operating conditions which normally obtain in the electron microscope. In their recent paper Zeitler and Bahr confine themselves to a comparison of Lippert's (23) experimental values of electron-scattering cross-sections with the Leisegang theory at objective apertures greater than 10^{-2} radian, in which elastic scattering is the predominant factor. They themselves state that the deviations from this theory at smaller apertures are due to inelastic scattering but, on the other hand, say that if apertures of 10^{-3} to 10^{-2} radian are used, the effect of inelastic scattering can be disregarded.

In view of this situation we have been concerned to re-state some of the main points of the current theories of electron scattering in relation to quantitative electron microscopy and to give some experimental values of electron-scattering cross-sections which may be of use in further comparisons between various scattering theories.

The main purpose of this paper, however, is to show how determinations of mass, density, and thickness may be made both for isolated specimens and, in principle, for regions of thin sections without recourse to theoretical contrast parameters. A quantitative examination has been made of the validity and accuracy of the methods described by a comparison between mass measurements made in the electron microscope and those made by other, well established, methods. A ram spermatozoon, calibrated by interference microscopy, provides an object at the upper limit of size practicable for electron microscopy while a bacteriophage, calibrated by sedimentation and diffusion measurements (31) provides a test object with dimensions well below the resolving limit of the optical microscope and in the "normal" electron microscope range. Measurements on both these objects are reported and, in addition, the change in mass of T2 bacteriophage after staining with uranyl acetate is estimated as an application of the method.

Measurements on the density of the nucleus and cytoplasmic membrane of ram sperm, as seen in thin section, have already been published (30).

Electron Scattering

The contrast in the electron microscope of an image of an effectively amorphous object (*i.e.* one in which the effect of coherent scattering is negligible) is due almost entirely to the differential scattering of electrons by various parts of the object, which causes varying fractions of the incident beam to be prevented from passing through the objective aperture of the microscope and contributing to the image intensity. Under normal operating conditions the contrast at a given point of the image is governed by the exponential relation (16),

$$I = I_o e^{-Sw} \tag{1}$$

where I_o is the intensity of the electron beam incident on the object of mass-thickness w, I is the intensity of the transmitted beam which reaches the image plane, and S is the effective total electron-scattering cross-section per gram of material. The mass-thickness $w = \rho t$ if ρ is the density of the material and t the thickness of the object in a direction parallel to the incident beam. The relation (1) has been verified experimentally by Hall (15) and Hillier and Ellis (19) and more recently by Coupland (7). For scattering by a single element the atomic cross-section σ is related to S by the formula

$$S = \sigma \frac{L}{A} \tag{2}$$

where L is Avogadro's number and A the atomic weight of the element. It should be pointed out, however, that it is assumed in equation (2) that the total scattering cross-section of a body is the sum of the elastic and inelastic cross-sections of its constituent atoms. In reality, additional effects are present due to the binding of atoms in molecules, to scattering by free electrons in the case of metals, and to coherent scattering in crystalline materials.

The expressions for the elastic scattering component, S_e, of S given by Leisegang (21) and Lenz (22) are similar in form and differ mainly in approximations to the effective radius, R, of the scattering atom. Leisegang uses a formula $R = a_o Z^{-1/3}$, where $a_o = 0.529$ A, which implies that $S_e \propto \frac{Z^{4/3}}{A}$ for the normal range of objective apertures. Lenz uses selected values of R which result in a smaller variation of S_e with Z. Lenz' expressions are given in a practical form by Sadhukhan (28) and tables showing the dependence of the cross-sections upon atomic number, aperture, and accelerating voltage are given by Cosslett (6).

Zeitler and Bahr, using Leisegang's theory, effectively put $S = S_e$ which means that S should increase with atomic number. While this is encouraging from the point of view of substance differentiation (5) it would mean that to determine accurately the mass-thickness of a substance in the electron microscope its chemical composition would need to be known beforehand. On the other hand, Lenz includes in S a contribution due to inelastic scattering, *i.e.* $S = S_e + S_i$, and shows theoretically that S_i is of the same order of magnitude as S_e for elements of low atomic number (*e.g.* carbon) and increases with decreasing aperture angle. The effect of the addition of the inelastic and elastic scattering components is that the theoretical value of S becomes almost independent of atomic number as shown by us (30) and by Cosslett (6) in his Table III. This implies that no *a priori* knowledge of a specimen is required for mass measurement and that, for instance, in measurements on an organic specimen the same value of S should be valid both before and after staining with heavy-metal ions. That S should be independent of Z is thus of fundamental importance to quantitative methods of measurement.

Recent evidence for the importance of the inelastic component of S has been given by Valentine (36) who has pointed out that the variation of S with objective aperture, α, is greatest at small values of Z (*i.e.* less than about 12) and that this fact may be used experimentally to distinguish between objects of low and high mean atomic number. Since the elastic scattering cross-section (predominant for high Z) varies very little with α, the variation of contrast observed at low Z must be due almost entirely to the variation of S_i. The magnitude of the effect observed by Valentine makes it probable that, at the smaller aperture he used, S_i was at least as large as S_e. An experimental determination of the variation of S with Z has lately been made by Reimer (27) who plotted the contrast $\left(\log \frac{I_o}{I}\right)$ of evaporated films against their mass-thickness. For a range of elements from C to U and a range of mass-thicknesses up to 40 μg./cm.^{2} he found that all the experimental points lay on a single straight line; *i.e.* for the conditions he used (60 kv. and an objective aperture of 4.6×10^{-3} radian) S was sensibly independent of Z, a fact which has already been reported by Hall (15) for an electron microscope in which no physical aperture was used.

Four experimental values of S for accelerating potentials of 25, 50, 75, and 100 kv. at the apertures quoted are given in Table I and compared with the calculated values for carbon. The value of S was determined from electron micrographs of polystyrene spheres of known mass-thickness, a method used by Hall and Inoue (18) whose corresponding results are shown. There is an order-of-magnitude agreement between S_{calc} and S_{obs} but the experimental values are systematically lower than the calculated ones; the discrepancy increases rapidly as the accelerating voltage decreases.

Theoretical values of S are calculated for single-scattering conditions in the absence of phase effects and lens aberrations. However, the distortion of the edge contrast of an image due to geometrical aberrations can be made negligible by using a suitable objective aperture; phase and diffraction effects are minimised at the in-focus position. It has been shown experimentally that the values of S determined from thick specimens in which plural scattering may be expected to occur are, within experimental error, the same as those determined for thin specimens. Thus Hall (15) found that the exponential relation (1) held for values of $\ln I_o/I$ up to 3.0, while Hall and Inoue (18) and Zeitler and Bahr (39) both publish experimental graphs of $\ln \frac{I_o}{I}$ against w which are linear up to $\ln I_o/I \simeq 2.4$. Bahr (2) suggests that the range of mass-thickness measurement can be extended by a factor of 6 to 7 upon his original estimate of the limiting value which was presumably based (38) on the equation $\ln \frac{I_o}{I} = 0.53$.

Cosslett (6) states that values of $\ln \frac{I_o}{I}$ up to 5 may be used with an accuracy of 5 to 10 per cent in S. Values of the calculated transparency thickness have been included in Table I on the basis of the von Borries (3) definition, $\frac{1}{S_e}$. The units have been chosen so that the numerical values show the thickness in A for a material of unit density.

Hall (17) found that the effective value of S varied slightly with the condition of focus of the image and this observation has been confirmed in the present work. In addition, S will depend to some extent on the convergence of the incident electron beam and on the electromagnetic field between the specimen and the objective aperture (7). Thus, to find the value of S obtaining during the exposure of a micrograph it is necessary for quantitative work to "calibrate" it by including objects of known mass-thickness. It is shown in the next section how the experimental value of S can be used in the determination of density and mass in the electron microscope.

TABLE I

Values of S

A comparison of values observed with 880 A diameter polystyrene spheres,* calculated values for C‡ and values reported by Hall and Inoue (18) which are shown bracketed

kv.	α radian $\times 10^3$	S_e cm.^{2} gm.$^{-1}$ $\times 10^{-4}$	$1/S_e$ gm. cm.$^{-2}$ $\times 10^3$	S_i cm.^{2} gm.$^{-1}$ $\times 10^{-4}$	S_{calc} cm.^{2} gm.$^{-1}$ $\times 10^{-4}$	S_{obs} cm.^{2} gm.$^{-1}$ $\times 10^{-4}$
100	2.56	3.21	3120	3.93	7.14	5.4 ± 0.1
[100	2.2					5.9]
75	3.49	4.20	2380	4.81	9.01	5.9 ± 0.2
50	3.68	5.91	1690	7.34	13.25	8.8 ± 0.2
[50	4.2					8.2]
25	2.94	11.15	897	17.98	29.13	13 ± 1

* The density of polystyrene was taken to be 1.05 gm. cm^{-3} (37) whereas Hall and Inoue used a value of 1.1 gm. cm.$^{-3}$.

‡ Lenz' expressions were used and R was assumed to have the value $0.529.6^{-1/3}$ A. Full relativistic corrections were made.

Object Models and the Measurement of Mass, Thickness, and Density

There are two main classes of electron microscope specimens for which quantitative measurements may be required. These comprise isolated specimens mounted directly on an electron microscope support film and thin tissue sections which are similarly mounted. These two object models are discussed below for the ideal case in which no sublimation or contamination of the specimen occurs.

Directly Mounted Specimens: Consider a small homogeneous region of a specimen of thickness t, density ρ, and area dA perpendicular to the incident uniform electron beam, mounted on a film which is of uniform thickness over the area A of the specimen. Let the intensities of the electron beams transmitted by the film and specimen together and by the film alone immediately adjacent to the specimen be I_2 and I_1, respectively. Then the mass of the specimen is given by

$$M = \int^A \rho t dA$$

$$i.e. \quad M = \frac{1}{S} \int^A \ln \frac{I_1}{I_2} dA, \quad \text{from equation (1).} \tag{3}$$

The above equation is the fundamental one by which the mass of a specimen may be determined and may be applied only if S is independent of atomic number and mass-thickness. That this independence may be achieved in practice has been shown in the previous section.

Experimentally, electron intensities are usually recorded photographically and equation (3) may be expressed in two forms depending on the precise conditions of photographic recording. If photographic densities above background, D_1 and D_2, on the plate are linearly related to I_1 and I_2 the equation becomes

$$M = \frac{1}{S}\int^{A} \ln \frac{D_1}{D_2}\, dA \qquad (4)$$

or, if D_1 and D_2 are proportional to log I_1 and log I_2, respectively, it takes the form

$$M = \frac{\gamma}{S}\int^{A} (D_1 - D_2)\, dA \qquad (5)$$

where γ is the photographic constant. Methods familiar in microspectrophotometry (4) may be used to perform the integrations above. Either equation (4) or equation (5) is suitable for the evaluation of the total mass of any isolated specimen providing that over its area variations in the thickness of the supporting film are not appreciable.

While the mass-thickness, ρt, is readily determined for any point on the specimen the density or thickness for this type of object may only be found indirectly. If ρ is known from the nature of the material or from macroscopic measurements then t may be calculated. Alternatively, for suitable specimens, t may be found by metal shadowing or stereomicroscopy, thus allowing the determination of ρ.

Thin Sections: A comparative method for the measurement of density in thin sections has already been described by us (30) but the main points will be mentioned here for the sake of completeness. Regions of sections, to be suitable for measurement, must be of a defined thickness, *i.e.* they must extend through the entire section thickness from the upper surface to the supporting film. To determine the mass, thickness, and density of such regions it is necessary to have a measure of the intensity of the electrons transmitted by the supporting film alone. A hole in the section sufficiently near the region of interest leaving the supporting film exposed cannot be expected to occur very often; however, if a reference object of known density is embedded with the specimen and the whole sectioned together, all three of the parameters M, ρ, t can be determined under favourable conditions.

Suitable reference objects will depend on the specimen and in many organic specimens will already be available in the section (although assumptions about the embedded densities of these objects may have to be made (compare (30)). It is possible to mix intimately reference particles in high concentration with some specimens, *e.g.* bacteria and spermatozoa. The particles should preferably be impermeable to the embedding medium (to retain a constant density) yet be readily embedded and sectioned with the specimen under examination. Small particles of plastic material (*e.g.* polystyrene latex spheres) or graphite ("aquadag") may be of use in this way; we have successfully sectioned colloidal graphite particles in methacrylate and polystyrene spheres in vestopal. The reference objects must be sufficiently near the regions of interest in the specimen for random variations of thickness in section and supporting film to be neglected. As pointed out in a previous paper (30) non-random variations of section mass-thickness (*e.g.* due to sublimation of the embedding medium) will introduce errors in the expressions for the density of the specimen if the measured and reference densities are not equal. In a recent paper Reimer (27) has shown that even in a carbon-formvar "sandwich" methacrylate loses about 30 per cent of its mass-thickness in the electron beam, while araldite and vestopal (unprotected) lose about 20 per cent and 13 per cent, respectively. The best embedding medium at present available for quantitative measurements is clearly vestopal, while suitable specimens will be those which on applying tests such as those used by Morgan, Moore, and Rose (25) show no differential sublimation.

EXPERIMENTAL

Calibration of the Electron Microscope: A Metropolitan-Vickers type E.M.3. electron microscope with electrostatic astigmatism correction was used at accelerating voltages of 25, 50, 75, and 100 kv. The semi-angular apertures of the objective lens used in this work were determined by using electron diffraction conditions (with the same objective lens current used in the determination of S by normal microscopy; see Table I) and observing the angle at which the diffraction pattern from a known salt was cut off by the aperture. The aperture thus determined is the effective aperture corrected for distortion of the electron paths by the field of the objective lens.

The magnification **M** and total mass-scattering cross-section S valid for each micrograph were determined by including polystyrene spheres of diameter 880 ± 80 A in each specimen. Errors in **M** occur because of the deposit of a layer of contamination over regions of the specimen which are irradiated by the electron beam (12) while errors in S may be caused both by the effects of contamination and by sublimation of material from the spheres, caused by the electron beam.

The rate at which the contaminating layer was deposited on a surface for the beam currents used in

this work (<6 mA cm.$^{-2}$ at the specimen) was found to be about 6 A per minute. The specimen area irradiated at any one time was kept to a minimum ($\backsim(50\ \mu)^2$) by using a screening aperture above the specimen similar to that described by Page and Agar (26). The screening aperture assembly was also used as a Faraday cage in order to measure the beam current (14). With the types of specimen used, *i.e.* mixtures of polystyrene spheres with ram sperm and of polystyrene spheres with bacteriophage, little time was lost in scanning the specimens for suitable areas and it is believed that the systematic error in the value of **M** introduced by contamination is not greater than +4 per cent. This error corresponds to about 3 minutes irradiation.

Polystyrene spheres are commonly and successfully used for magnification calibration and the values of **M** measured in this work using the spheres were in good agreement with those found using a replica of a diffraction grating. The effects of sublimation, if this occurs, must, therefore, be confined to the reduction in density of the spheres without change in radius. The possibility seemed to us to be small since Hall (15) and Hall & Inoue (18) had determined S under carefully defined experimental conditions using polystyrene spheres. It is also known that polystyrene is stable in the absence of oxygen up to a temperature of 250°C. (34); this specimen temperature is not reached in the electron microscope except possibly under the most extreme conditions (16). However, the possibility of sublimation was examined by mounting some polystyrene spheres on an evaporated carbon film of known mass-thickness. The mass-thickness of the carbon film, obtained by weighing, was 6.60 ± 0.40 μg. cm.$^{-2}$. The mass-thickness of the carbon film was measured from electron micrographs (beam current at specimen 6 mA cm.$^{-2}$, accelerating voltage 75 kv.) using the mean value of S determined from measurements on 12 polystyrene spheres (for method of measurement see below) taking the density of polystyrene to be 1.05 gm. cm.$^{-3}$ (37). The electron microscope value for the mass-thickness of the carbon film was 6.77 ± 0.23 μg. cm.$^{-2}$. It was, therefore, concluded that under the experimental conditions used for mass measurement the sublimation of polystyrene is negligible.

S may be determined in three ways, from measurements based on the following equations (assuming, for the moment, no contamination).

$$S = \frac{1}{2\rho R} \ln \frac{I_1}{(I_2)_{\min}} \tag{6}$$

$$S = \frac{1}{\pi\rho R^2} \int_o^{2R} \ln \frac{I_1}{I_2}\, dl \tag{7}$$

$$S = \frac{3}{4\pi\rho R^3} \int_o^{\pi R^2} \ln \frac{I_1}{I_2}\, dA \tag{8}$$

where the density of polystyrene (ρ) is 1.05 gm. cm.$^{-3}$ (37) and the spheres are of radius R.

In equation (6), I_1 is the intensity transmitted by the clear film and $(I_2)_{\min}$ is the intensity transmitted at the centre of the image of the sphere where the electron path length in the sphere is $2R$. In equation (7), account is taken of the variation of I_2 across a diameter of the sphere and in equation (8), of the variation in I_2 over the projected area of the sphere. Application of equation (6) assumes spherical symmetry of the particle and no distortion, *e.g.* flattening, during specimen preparation and examination. Equation (7) assumes rotational symmetry of the particle about a line through its centre perpendicular to the supporting film. Equation (8) makes no assumptions.

When a layer of contamination (density $f\rho$ and thickness r) covers both the sphere and the supporting film, I_1 and $(I_2)_{\min}$ (equation 6), are reduced in the same ratio. The value of S obtained using equation 6 is thus independent of the effect of contamination but assumes an undistorted particle.

Measurements of S were made for polystyrene spheres of 1500 A and 2700 A diameter using equations (6) and (7). The values of S for these spheres indicated that they were flattened by up to 10 per cent. In the case of 880 A diameter spheres it was difficult to distinguish the effects of distortion from those due to contamination.

To correct for possible distortion of the spheres either equation (7) or (8) may be used in a suitable form corrected for the presence of the layer of contamination. The modified form of equation (7) is

$$S\left[f(1-\theta/\pi) + \frac{1-m}{1+m}\frac{\sin\theta}{\pi} + \frac{1-f}{(1+m)^2}\right] = SF = \frac{1}{\pi\rho(R+r)^2}\int_o^{2(R+r)} \ln\frac{I_1}{I_2}\, dl \tag{9}$$

where

$$\sin\theta = \frac{2\sqrt{m}}{1+m} \quad \text{and} \quad m = r/R.$$

The right hand side of this equation may be evaluated experimentally giving the value of the product SF but not S directly in the absence of a very carefully standardised contamination rate. In practice the polystyrene spheres are assumed to have a radius R, since in the absence of a second means for measuring the magnification, r in any given micrograph cannot be determined. The experimental integral which is evaluated is therefore

$$S' = (1 + m)SF = \frac{1}{\pi\rho R^2}\int_o^{2R} \ln \frac{I_1}{I_2}\, dl \qquad (10)$$

The point at issue here is how different is S' from S for given experimental conditions? Substituting $f = 2$, *i.e.* assuming the density of the contamination is approximately that of evaporated carbon, gives the following values of $(m, S'/S)$—(0.01, 0.96), (0.05, 0.98), (0.07, 1.00), (0.10, 1.03), (0.20, 1.11). A variation of this form has been observed experimentally for 880 A polystyrene spheres.

The variation of S with variation in objective lens current has been examined semi-quantitatively by Hall (17). A quantitative analysis of this effect was made by taking a through-focus series of polystyrene spheres at a magnification of 40,000 at 100 kv. using an objective aperture of 5×10^{-3} radian. In the range between $\pm 1\ \mu$ from focus S was found to be constant within 2 per cent. Micrographs with greater off-focus distances were not used for analysis.

It is considered unlikely that any of the micrographs used in this work correspond to $m > 0.04$, giving $S'/S \backsim 0.97$. The measurement of mass depends on the ratio $\frac{1}{\mathbf{M}^2 S}$ and it is important to assess the effect of contamination on this ratio. If a number of micrographs are used for analysis with fractional changes in magnification due to contamination lying between $(1 + m_1)$ and $(1 + m_2)$ corresponding to different times of irradiation, then the mean mass $\bar{M}$ measured for a particle of "true" mass M is

$$\bar{M} = \frac{M}{(m_2 - m_1)}\int_{m_1}^{m_2} \frac{dm}{(1 + m)^2} = \frac{M}{(1 + m_1)(1 + m_2)}$$

if equation (6) is used to determine S. Taking $m_1 = 0.01$ and $m_2 = 0.04$, which are representative of the conditions used while taking the ten micrographs of ram spermatozoa (see next section), gives $1.05\ \bar{M} = M$, *i.e.* the mean result is subject to a systematic error of about -5 per cent due to magnification errors.

Values of M calculated from a single micrograph using equation (10) in the determination of S are subject to a maximum systematic error due to contamination of about -5 per cent. This is the case for the measurements made on bacteriophage (see next section).

Photographic Methods: It was shown in the previous section that the equations for mass could take two forms according to the conditions of photographic recording. It was decided to make use of the region of the photographic-plate characteristic where the optical density was directly proportional to the incident intensity (corresponding to equation (4)) rather than the linear region of the *H-D* curve (corresponding to equation (5)). This course was taken since experience has shown that the first condition may be readily achieved by taking micrographs on Ilford special lantern contrasty plates followed by standard processing to give optical densities less than 1.5. The linear region of the *H-D* curve occurs at higher densities (1.7 to 3) and it is more difficult to maintain a constancy of γ from plate to plate.

The procedure adopted in using equation (4) was to take a set of densitometer traces along equally spaced parallel lines across the image of the specimen using either a "linear" or an "exponential" (29) optical wedge and a double-beam recording microdensitometer (32). Optical densities were measured above the photographic density corresponding to a specimen-grid bar following the method of Hall (15). The method of strip-wise integration is familiar in quantitative analysis by optical methods (4). The integration corresponds to a determination of the volume beneath a mass-thickness "profile" of the specimen as illustrated in Fig. 1 for a bacteriophage particle. The traces shown were made with the "exponential" wedge.

Specimen Preparation: The specimens of whole ram sperm were prepared by allowing drops of a dilute suspension of sperm and 880 A diameter polystyrene spheres in 70 per cent alcohol to evaporate on specimen supporting films. Specimens of unstained T2 Lr^+h^+ bacteriophage were prepared similarly from suspension in isotonic salt solution. Specimens of bacteriophage were stained with 4 per cent uranyl acetate for 1½ hours and dialysed against a large volume of glass-distilled water for 12 hours.

RESULTS

Mass of the Heads of Whole Ram Sperm

Whole ram sperm do not make ideal specimens for electron microscopy since the dimensions of the head (length, width, and thickness about 6 μ, 3 μ, and 0.3 μ respectively) are such that the head fills the microscope field at a magnification of about 8000 times; the thickness difficulty was largely overcome by using an accelerating voltage of 100 kv. At this voltage the value of $\ln I_o/I$ for a ram sperm head was about 2 which is well within the range, discussed already, where S is independent of w. Such a large specimen was required to overlap the range of measurement of the interference microscope.

The masses of the heads of ten whole sperm were determined using equation (4). The mean values of $\mathbf{M}$ and S, the latter using equation (6), were determined from measurements on the polystyrene spheres included with the sperm. The mean magnification (including the effect of

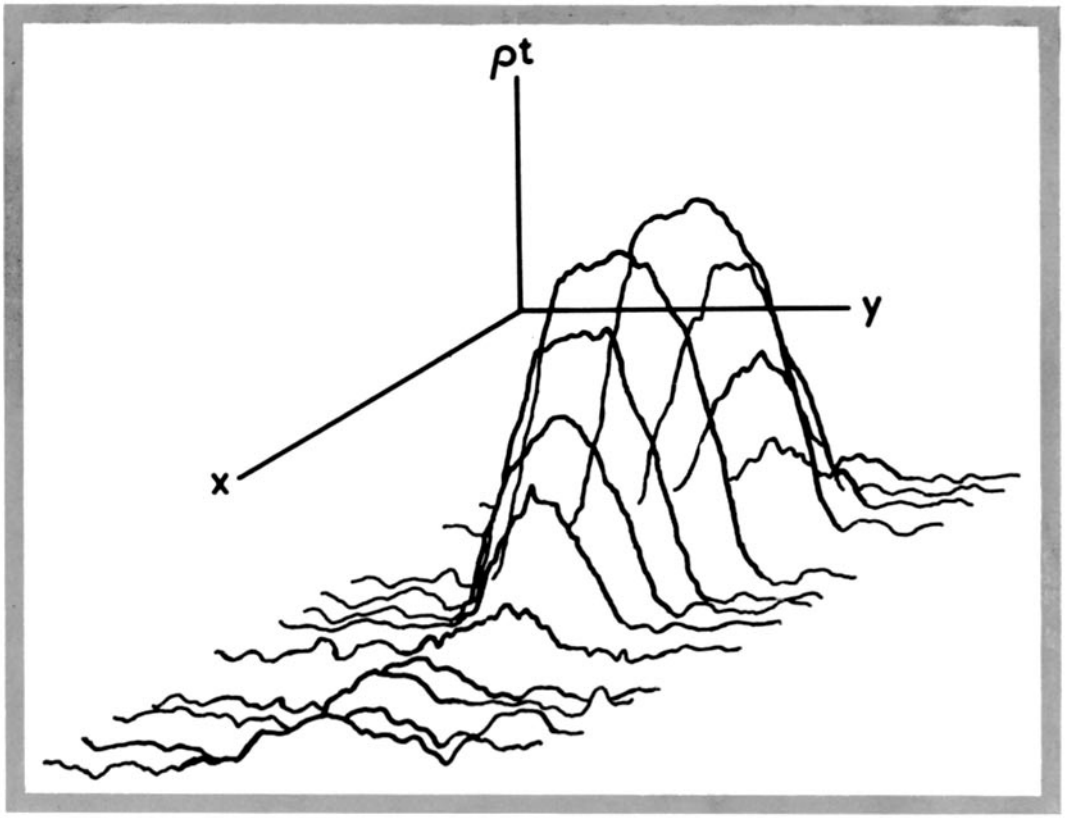

FIGURE 1
Three-dimensional isometric projection of the mass-thickness profile of a T2 bacteriophage. The tail of the phage extends in the x direction. The lengths of the x and y axes represent 1000 A and that of the mass-thickness axis (ρt) corresponds to 1000 A for $\rho = 1$ gm. cm.$^{-3}$.

contamination) was 8250 ± 120 (from 34 spheres) and the mean S was 4.37 ± 0.09 × 10^4 cm.^{2} gm.$^{-1}$ (from 38 spheres). The mean mass of a ram sperm head determined by electron microscopy was 7.74 ± 0.42 × 10^{-12} gm.; this value may be low by about 5 per cent due to the effect of contamination as already discussed.

Mass of the Heads of T2 Lr^+h^+ Bacteriophage

Micrographs were taken at 75 kv. at a magnification of 34,800 ± 610. This value includes the effect of contamination. The value of S obtaining was 5.77 ± 0.08 × 10^{-4} cm.^{2} gm.$^{-1}$; S was determined using equation (10). Both **M** and S were determined by measurements on 10 polystyrene spheres. The mean mass of the heads of the bacteriophage (ten measurements) was found to be 3.00 ± 0.14 × 10^{-16} gm. This value is probably low, due to the effects of contamination, by up to 5 per cent. If the head is assumed to be hexagonal in section its mean density is 1.74 ± 0.10 gm. cm.$^{-3}$ (this value may be high by up to 3 per cent due to errors in S only).

Wide variations were found in the measurements of the masses of phage heads stained with uranyl acetate. It was apparent that the conditions used during staining were destructive and a number of the phage heads were either partly or completely "ghosted" through the loss of deoxyribonucleic acid (DNA). Mass measurements were made on 25 stained phage heads and values of mass below the mean unstained value were rejected. The mean increase of mass of the heads due to staining was found to be about 45 per cent.

DISCUSSION

The assessment of the validity of a new experimental method depends on the agreement between similar measurements made by the new

method and by well established ones. In this work two comparable sets of results are presented.

The value of $7.74 \pm 0.42 \times 10^{-12}$ gm. found for the mean mass of a ram sperm head is to be compared with the mean mass of $7.13 \pm 0.29 \times 10^{-12}$ gm. found by interference microscope measurements on ten similar heads (30). The electron microscope value is very probably low by up to 5 per cent due to the effects of contamination on **M**. The layer of contamination which builds up over the surfaces of the sperm heads will have little effect on the measured mass since its effect will be to increase the thicknesses of the specimen and the supporting film at (presumably) the same rate. The mass of the layer of contamination is, in any case, small compared with the mass of the sperm head.

The mass of the head of a T2 bacteriophage found by electron microscopy is $3.00 \pm 0.14 \times 10^{-16}$ gm. while Taylor, Epstein, and Lauffer (31) found values of 3.65×10^{-16} gm. and 3.01×10^{-16} gm. at pH 5 and 7, respectively, from the combined results of sedimentation and diffusion measurements. The latter two values include the mass of the tail of the bacteriophage particle and are absolute, *i.e.* do not depend on an assumed shape for the particle. The tail has the dimensions 250×1000 A (35) and is associated with about 10 per cent of the mass of the particle. The electron microscope value for the mass of the phage particle is again very probably low by up to 5 per cent due to the effects of contamination on **M** and S. However, it should be noted that the method adopted in the measurement of mass using polystyrene spheres in order to determine S involves essentially a *comparison* of the mass of a sphere with that of the "unknown" particle. When the two objects being compared are very similar in linear dimensions, as in the present case, the effect of contamination on the measurement of mass will, to a large extent, cancel out. The mass of an *uncontaminated* phage head should, therefore, providing the rates of contamination of the two specimens are comparable, be very close to the value obtained without applying corrections for contamination, *i.e.* $3.00 \pm 0.14 \times 10^{-16}$ gm. The value for the density of the material of a phage head evaluated on the assumption that the head is undistorted on drying is 1.74 ± 0.10 gm. cm.$^{-3}$ (a value which includes a systematic error of up to 3 per cent) and may be compared with the density of dry DNA, *i.e.* about 1.65 gm. cm.$^{-3}$.

We believe the reasonable agreement between these electron microscope values for mass and density and those found by other methods to be most encouraging and that under carefully controlled conditions the electron microscope can provide quantitative results. This agreement, together with the demonstration that polystyrene does not sublime at low beam currents, gives reasonable evidence that the principal component materials of the specimens used do not sublime in the electron microscope under the same conditions. The greatest systematic error to which the given measurements are subject is due to the effect of contamination on the value of **M**. This error could be reduced or eliminated by using very short exposure times or by measuring **M** by a method involving the separation of points.

The present unanimity of both experimental results and theoretical prediction that S is essentially independent of Z for the usual operating conditions in electron microscopy gives good reason for the supposition that quantitative measurements of the uptake of electron stains by biological specimens, exemplified in this work by the measurements on stained bacteriophage, are valid within the present accuracy of measurement.

Quantitative measurements on thin sections are with present embedding media subject to error because of the sublimation of the embedding medium itself and because of the differential sublimation of specimen material. The latter may, in fact, be due in some cases to the differential loss of embedding medium rather than of material from the specimen itself but this is of course no less serious.

The Minimum Mass Measurable by Electron Microscopy

The minimum mass depends on the minimum linear dimensions in the plane of the supporting film for which phase and edge distortion effects are small and on the accuracy with which optical densities may be measured. The minimum dimensions will vary from instrument to instrument and will be a function of the coherence of the incident electron beam (13) and the various lens aberrations. For the present purpose a flat disc of 400 A diameter in the plane of the supporting film will be considered. The smallest measurable density difference $(D_1 - D_2)$ is a function of the smoothness of the supporting film, care taken in specimen preparation, grain in the photographic

plate, uniformity of illumination, and the desired accuracy of measurement. The effect of contamination will be very important when small particles are considered but can be minimised either by short exposures of the specimen to the electron beam or possibly by using a specimen stage cooled to an appropriate temperature (12).

For small objects the fundamental limitation (neglecting contamination) will be in the measurement of D_1 and D_2 since these values will be almost equal: for this case equation (4) becomes

$$M = \frac{1}{S} \int^{A} \frac{D_1 - D_2}{D_2} dA \qquad (11)$$

If the random errors in D_1 and D_2 are equal and of value x then the fractional error in $\frac{D_1 - D_2}{D_2}$ is $\frac{2x}{D_2 - D_1}$. If a 5 per cent error in the density ratio is allowable on a single measurement, equation (11) becomes, for an object of constant thickness and density,

$$M_{\min} = \frac{40x}{SD_2} \int^{A_{\min}} dA \qquad (12)$$

A typical value of x/D_2 is 1/200 and for a flat disc of 400 A diameter the minimal measurable mass (to an accuracy of 5 per cent on a single measurement) is about $\frac{2.5 \times 10^{-12}}{S}$ gm. For 75 kv. electrons the minimum mass is about 5×10^{-17} gm. corresponding to a minimum specimen thickness of 400 A for a material of unit density; from our results equivalent values for 25 kv. electrons would be about 2×10^{-17} gm. and 160 A, respectively. For an accuracy of 10 per cent these figures are halved.

Measurements of mass on the basis of equation (5) are necessarily less accurate than those based on equation (4) since the photographic plate in the γ region is less sensitive (10) to changes in image intensity.

Mass measurements at low accelerating voltages should be more accurate (for a given specimen) than measurements made at higher voltages, and measurements should be possible on thinner specimens. However, for these advantages to be realised it is necessary that the resolution of the microscope at the lower voltages should be as good as that normally achieved at the usual operating voltages of 50 to 100 kv. It also appears from the trend of the electron-scattering cross-sections shown by our measurements that the very large increase in contrast currently hoped for at low voltages may not occur.

APPLICATIONS

The quantitative methods developed here may be used under carefully controlled conditions to measure the mass, thickness, and density of suitable objects (the main restriction being that specimens should not sublime in the electron beam). The mass of an object may be important of itself, or its rate of change with time or its change with chemical or physical treatment may be of interest. As in other cytochemical methods an automatic scanning technique may be desirable for the integration of equation (4) in routine measurements on inhomogeneous biological materials. The following examples are illustrative of the range of application.

1. The direct determination of molecular weight by individual measurements (lower limit about 10^7 at 25 kv. on the conservative estimates of the previous section) in contrast to the statistical average values found by other methods, *e.g.* ultracentrifugation and light scattering.
2. The quantitative estimation of the uptake of electron stains by isolated specimens and regions of thin sections, and an evaluation of the selectivity of such staining.
3. The determination of specimen composition by mass measurements before and after chemical extraction of a given component of the specimen.
4. The determination of specimen thickness and shape and the thickness of thin sections.

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