A Quantitative Estimation of the Uptake of Two New Electron Stains by the Cytoplasmic Membrane of Ram Sperm

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ABSTRACT

From microdensitometer measurements on electron micrographs of sectioned sperm heads it has been found that the electron stains, triiodobenzoyl chloride, and triiodophenyl*iso*cyanate, increase the image contrast of the cell membrane above its immediate background by about 40 per cent and 70 per cent respectively, while the nucleus remains unstained. Assumptions based on current electron scattering theory have been used to deduce the uptake by weight of the stains in terms of the density of the nucleus, which was estimated from complementary measurements made with the interference microscope and electron microscope. The uptake of the stains was found to be about 7 per cent and 12 per cent by weight respectively. It is suggested that the method used in this work could be applied generally for the density measurement of cell structures unresolved by the light microscope.

INTRODUCTION

The use of stains of high electron-scattering power on biological material examined in the electron microscope has been recognised for some time as a useful technique for improving the contrast in the image (26, 28, 32), and it has been the hope that a range of selective stains might become available in this field in a similar manner to the development of cytochemical methods in light microscopy. Osmium tetroxide has become accepted as one of the best fixatives and stains for electron microscopy but, unfortunately, although this subject has been examined by a number of authors (1, 2, 10, 11, 29, 30) there is still no general agreement on the specificity of the stain and the significance of its effect on the image contrast is open to question (27).

Consequently, there has been a search for other stains which might at the same time possess the properties of specificity for a compound of biological interest and a high electron-scattering cross-section per molecule. One of the first attempts in this field was by Lamb *et al.* (22) who used the dinitrofluoro-benzene reaction for protein and stained the mid-pieces of whole bull sperm with silver and lead salts. Bradfield (8) has used a modified Feulgen technique for the deposition of silver in bacterial nuclei, and in conjunction with Gibbons (12) has used lanthanum and thorium nitrates for the differential staining of locust spermatids. Barrnett and Palade (3) have located the sites of dehydrogenase activity in animal tissues by the precipitation of reduced potassium tellurite, and recently Watson (36, 37) in staining tissue sections has found uranyl acetate to be particularly successful in enhancing contrast, although for the staining of collagen he finds phosphotungstic acid still to be the most effective. (Whether the success of the latter is due purely to its staining properties is however open to doubt (20, 21)). It has also been suggested from considerations of electron-scattering theory that optical microscope stains should be effective in the electron microscope (18, 34).

Despite the volume of published work on electron staining, very few quantitative experimental results have been reported. In the present work the efficiency of two new staining preparations for electron microscope specimens has been investigated and a quantitative method has been developed for measurement of the uptake of stain, which is based on absolute measurements of mass-thickness in the interference microscope and relative measurements of density from electron

micrographs. In this method microdensitometer measurements made on electron micrographs of biological sections enable the density of detailed structures (*e.g.* sperm cell membranes) to be determined in terms of the density of larger areas in the section (*e.g.* nuclei) independently of variations in the thickness of the section or its supporting film. The density of the larger areas may be determined with the interference microscope by observing material of known thickness, and thus the density of structures unresolved by the light microscope may be found absolutely. In this way the effect of stains on the structures may be estimated quantitatively, and the percentage uptake by weight be determined. The interpretation of the results depends on several assumptions about the mechanism of image formation in the electron microscope and the form of the relevant expressions for electron-scattering cross-sections. In the next two sections the justification for these assumptions is discussed in the light of current electron-scattering theory, and the formation of an image is considered for an object model relevant to the experimental conditions.

Electron Scattering

Ever since the earlier treatments of the theory of image formation in the electron microscope (7, 25), it has been of particular interest in this field to apply the results of general electron-scattering theory to the special conditions encountered in the instrument (5). These conditions, *i.e.* accelerating potentials of the order of 100 kv. and very small scattering angles, were investigated experimentally by Bibermann *et al.* (4), and later by Hall (14) and Hillier and Ellis (17) who confirmed the basic scattering law,

$$I = I_0 e^{-Sw}$$

in which I_0 is the intensity of the electron beam incident on a specimen of mass thickness w, I is the intensity of the transmitted beam, and S is the effective scattering cross-section per gram of material. The cross-section for one atom, σ, is related to S, in the case of scattering by a single element, by the formula

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

in which L is Avogadro's number and A the atomic weight of the element.

For the purpose of a quantitative investigation of electron staining it is essential to know how the atomic cross-section varies with the atomic number, Z, of the scattering element. Leisegang (23) and others have derived expressions for the elastic scattering cross-section which show that σ_{elastic} is proportional to $Z^{4/3}$; a somewhat similar variation is given by Lenz (24) who inserts in the scattering formula of Wentzel (38) numerical constants calculated from the self-consistent-field theory of Hartree. The expressions given by Lenz agree with the results of Bibermann *et al.* (4) and have been compared favourably with the results of experiment by several later authors (13, 16, 19). Hall (15) found that in practice in the electron microscope the effective atomic cross-section increased linearly with Z, and thus proposed that S should be regarded as an instrumental constant. Recently Hall and Inoue (16) using polystyrene spheres as scattering objects have determined values of S for different accelerating potentials and objective apertures, and obtain values for the effective scattering cross-section of carbon which are close to those of the corresponding elastic cross-section given by Lenz. It is interesting to note that although the diameters of the spheres used were several times the "transparency thickness" for carbon (*i.e.* $p > 1$ in the notation of Lenz (24); $m > 1$ in that of Zeitler and Bahr (39)) no significant effects of multiple scattering were apparent. A calculation similar to that performed by Zworykin *et al.* (40) shows that this is to be expected, and raises the question whether the limitations proposed by Zeitler and Bahr (39) on the maximum mass-thickness for which multiple scattering may be neglected are not too rigorous, at least for conditions normally obtaining in the electron microscope.

In the present work only areas much larger than the resolving power of the electron microscope have been of interest, and thus phase and interference effects were not significant (6, 33). Single scattering only has been considered since section thicknesses were less than the "transparency thickness" for carbon. The elastic and inelastic atomic cross-sections for single scattering have been calculated (31) from Lenz' expressions, for the particular conditions used in this work (*i.e.* an accelerating potential of 75 kv. and an angular semi-aperture of the objective lens of 5×10^{-3} radian). The corresponding cross-sections per gram, S, were calculated using the simplifying assumption that $A = 2Z$. The vari-

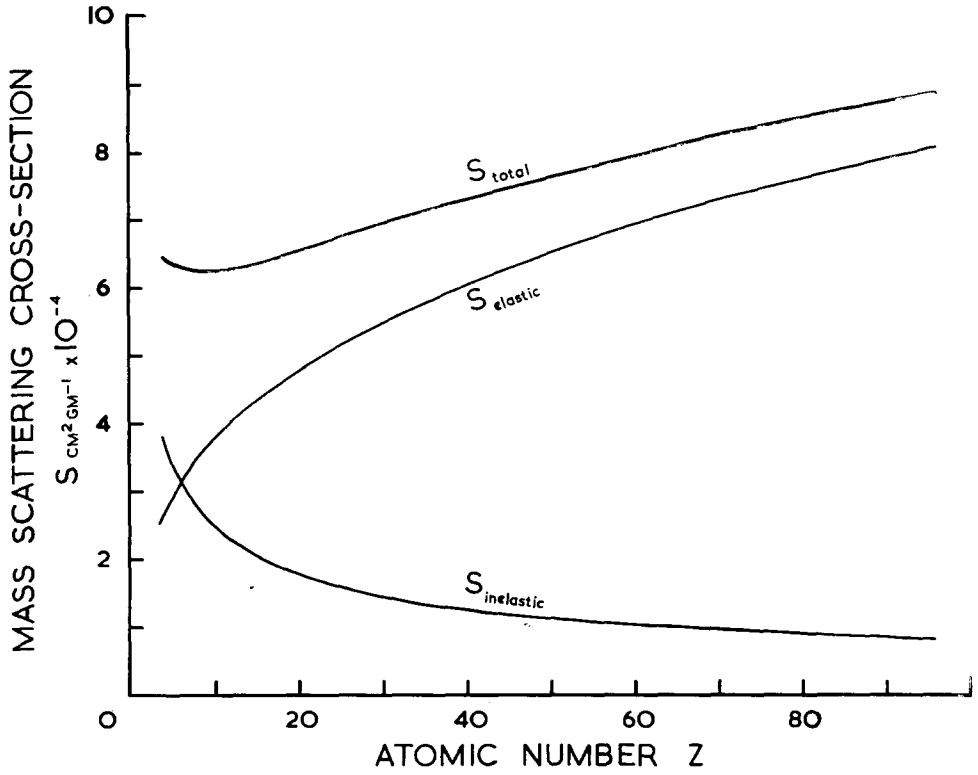

FIG. 1. Variation of mass scattering cross-sections for electrons with the atomic number of the scattering element. Values are calculated from Lenz' expressions (24) for an accelerating potential of 75 kv. and an objective semi-aperture angle of 5×10^{-3} radian. The simplifying assumption is made that $A = 2Z$.

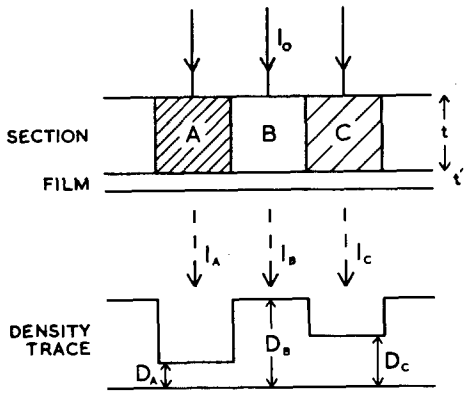

FIG. 2. A simple object model for a section in the electron microscope and the corresponding microdensitometer trace from an electron micrograph. For definition of symbols, see text.

ation of the cross-sections with Z is shown in Fig. 1. It can be seen that the elastic cross-section itself varies very little over the whole range of the periodic table. Thus even if only elastic processes were of importance in image formation, it would be a justifiable first-order approximation to make S a constant, independent of Z. If inelastic scattering is also taken into account, the total cross-section varies still less with the atomic number (15). In the treatment given below, S is throughout assumed to be a constant dependent only on the operating conditions in the electron microscope.

Application of Theory to a Particular Object Model

A simple object model which represents a section of an embedded specimen resting on a supporting film is shown in Fig. 2. Here all parts of the section within the region of interest are assumed to have the same thickness, t. If I_0 is the intensity of an electron beam incident normally on the section which contains homogeneous regions A, B, C of density ρ_A, ρ_B, ρ_C, respectively, then, for example, the intensity I_A at the image plane of the microscope, corresponding to region A, will be given by

$$I_A = I_0 \exp [-S(\rho_A t + \rho' t')] \quad (1)$$

in which S is the effective scattering cross-section per gram and ρ', t' are the density and thickness of the supporting film. If to register the intensity a photographic plate is used which exhibits a linear relation between exposure and photographic density, then on the developed plate the density, D_A, above background corresponding to region A (see Fig. 2) will be given by

$$D_A = kI_0 \exp [-S(\rho_A t + \rho' t')] \quad (2)$$

in which k is a constant. Similar expressions will hold for D_B and D_C. The expression (2) is inconvenient for the experimental determination of ρ_A because of the large number of variables involved, many of which it would be difficult to measure accurately. However if the ratio D_A/D_B is used, in which B, say, is a region of clear embedding medium, four of the variables need be considered no longer if it is assumed that they are the same for both regions; then

$$D_A/D_B = \exp [-St(\rho_A - \rho_B)]. \quad (3)$$

Now if a region C in the section has a known density, it may be used as a "standard scatterer" with which A may be compared, *i.e.* since

$$D_C/D_B = \exp [-St(\rho_C - \rho_B)] \quad (4)$$

then

$$\frac{\ln D_A/D_B}{\ln D_C/D_B} = \frac{\rho_A - \rho_B}{\rho_C - \rho_B} = Q, \text{ say.} \quad (5)$$

If $\ln I_B/I_A = \ln D_B/D_A$ is defined as the image contrast of region A with respect to clear embedding medium (B), Q then becomes simply the ratio of the contrast of A to that of C in the image of the section. From a single micrograph of a portion of the section containing a "standard scatterer" near the region of interest values of D_A, D_B, and D_C can be found by microdensitometry, and thus Q may be determined. The density ρ_B of the embedding medium can also easily be found. If ρ_C, the density of the standard scatterer is measured by an independent method, then the only unknown in the expression for Q is ρ_A. If measurements of D_A, D_B, and D_C are made very close together in the section, the assumptions made above of uniform thickness of the section and its supporting film and uniform beam intensity need only be valid over a small area of the specimen and effects due to the random fluctuation of these parameters across the object are correspondingly minimised. The absolute thickness of the section need not be known in the treatment above; it is sufficient to know that the section is thin enough for the effect of multiple scattering to be negligible (see section on electron scattering).

In the work described below the theory outlined in this section was used to determine the density of ram sperm cell membranes before and after staining of the whole sperm. Since it was expected that the sperm nucleus might not be affected by the stains, and, in fact, this appeared to be the case on examination of electron micrographs, the nucleus was designated a "standard scatterer" in the meaning given previously and its density was found separately using another method.

Materials and Methods

The biological material used in this study was ram sperm which was very kindly provided by Dr. A. Walton of the Agricultural Research Council Animal Research Station at Cambridge. The material is easy to embed and section, and because differences between individual sperm are small the material yields samples which are fairly homogeneous and thus suited to statistical analysis. The stains used were 3:4:5 triiodobenzoyl chloride and 3:4:5 triiodophenyl*iso*cyanate. These stains were produced by M. M. Coombs, J. F. Danielli, F.R.S., and P. A. Kendall, who kindly provided samples of stained sperm.

Electron Microscopy:

Samples of ram sperm which had been previously treated with the stains and control samples which had been fixed in absolute alcohol were centrifuged at about 1500 *g.* for 2 minutes. The supernatant was discarded and after dehydration and embedding in a mixture of 85 per cent butyl- and 15 per cent methylmethacrylate, the sperm were centrifuged again in the gelatin capsules prior to polymerisation of the medium. Prepared blocks of the polymer were cut on a thermal-advance microtome with a glass knife, and sections of about 500 A thickness were placed on copper specimen grids covered with carbon supporting films. Specimens were examined in a Metropolitan-Vickers EM3 electron microscope using a 50 μ diameter objective aperture, and later in an EM3A with the same objective aperture and focal length.

Micrographs of sections of the sperm heads were taken at magnifications of 9,000 (EM3) and 10,000 (EM3A). The photographic plates (Ilford Special Lantern, contrasty) were processed under standardised conditions in which they had been found to give a linear density/exposure relation over the range of photographic density 0 to 2.0. All the densities used in the calculation of results were within these limits. Using a micro-densitometer with a circular scanning aperture (35) traces were taken from the

micrographs in the way shown in Fig. 3. Measurements were made of the density above background corresponding to the nucleus, the cell membrane, and the methacrylate embedding medium. (Two measurements each were made for the membrane and methacrylate, one on each side of the nucleus, and a mean value recorded.) The size of the scanning aperture was chosen to be smaller than the width of the cell membrane on the micrograph. Measurements were made for control and stained samples, each of which contained from 50 to 100 sperm.

For each sperm head photographed a value of the ratio Q was calculated, *i.e.*

$$Q = \frac{\ln D_M/D_B}{\ln D_N/D_B}$$

in which suffices N, M, B denote nucleus, membrane, and methacrylate respectively. For each sample the frequency distribution of Q was found by noting the number of values, f, occurring within each interval of 0.1. To smooth out the effects of random errors,

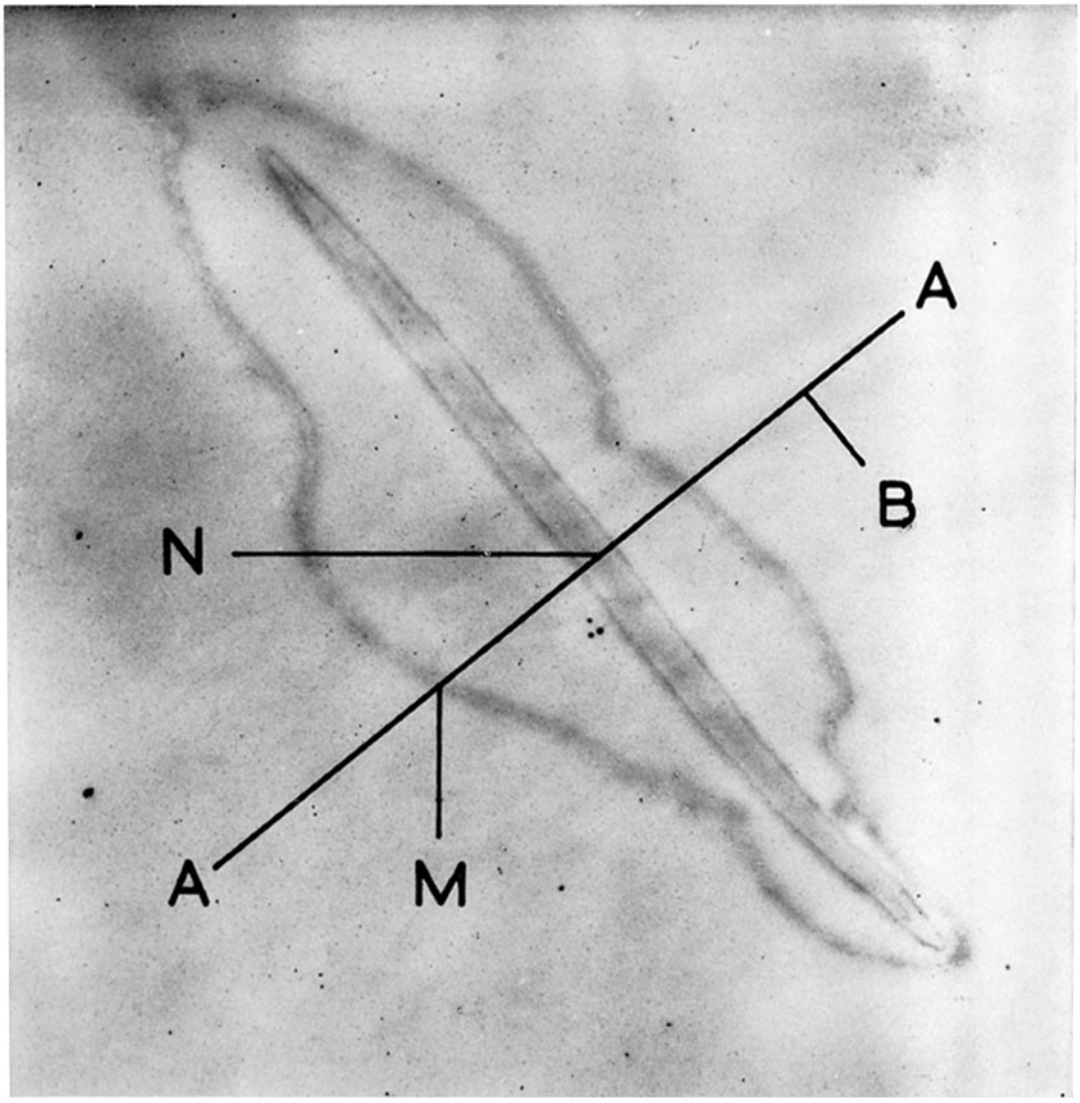

FIG. 3. Electron micrograph of a transverse section of a ram sperm head. AA denotes the line along which densitometer traces were made. Measurements of photographic density were made at points such as B (methacrylate), M (membrane), and N (nucleus). × 21,000.

the average value of f was found for each successive group of four intervals. This moving average was normalised and plotted at the centre of each group. The effect on Q of the sublimation of the embedding medium was checked and is discussed later.

In addition to the density measurements on the micrographs, measurements were made of the thickness of each nucleus as seen in transverse section. It was found that a high degree of orientation existed among the sperm heads, due probably to the centrifugation of the sperm prior to polymerisation of the embedding medium. The standard deviation of the thickness measurements indicated that the inclination of the sections to a true transverse section was in general quite small, and no correction was made for random variations in the section angle. A mean thickness was calculated for each sample. The average density of blocks of the polymerised embedding medium was determined with a specific gravity bottle.

Interference Microscopy:

The dry masses of whole sperm heads were found in the following way. Preparations of whole sperm in glass-distilled water were observed by monochromatic light of wavelength 546 mμ in a Baker interference microscope with an optical wave-shearing system and ×100 objective. The Baker "half-shade eyepiece" was used to determine the average phase difference, θ, between light which had passed through a sperm head and that which had passed through the distilled water. (The half-shade eyepiece is a photometric device which is not dependent on any external light source for comparison purposes, but which is used simply to compare an image of the specimen with part of itself on which an artificial constant phase shift has been imposed. Because of this it may be shown that absorption of light by the specimen has no effect on the phase measurement.) A photomicrograph was taken of each head for which a phase measurement was made, so that the corresponding projected area might be determined. Two independently prepared samples were examined from each of the stained materials and from the control; ten sperm heads were measured in each sample.

The dry mass, M, of a sperm head was calculated using the formula

$$M = \frac{A}{\chi} \cdot \lambda \cdot \frac{\theta}{2\pi}$$

in which A is the projected area of the head in a plane perpendicular to the incident light of wavelength λ, and χ is a constant for most cell materials (9) and was taken to be 0.18 cm.^{3} gm^{-1}. The mean dry mass of a sperm head was found for each sample. The density was calculated using the mean area of the heads

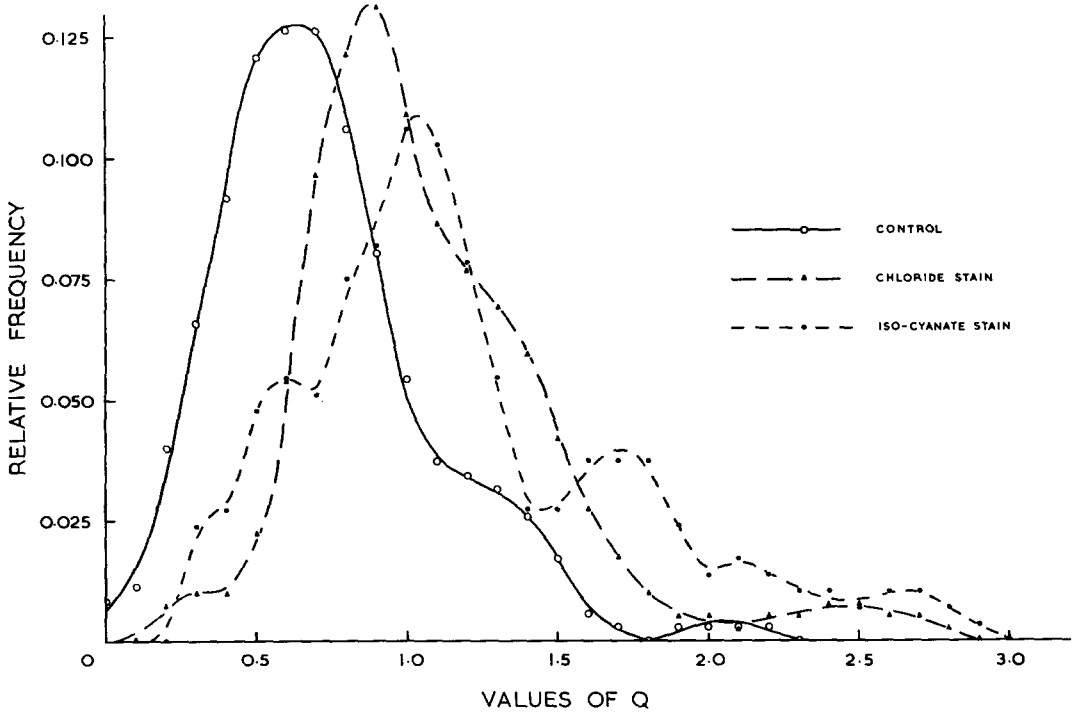

FIG. 4. Normalised frequency distributions of values of Q. A moving average has been used to smooth out random errors (see text). "Chloride stain" refers to sperm treated with triiodobenzoyl chloride and "*iso*cyanate stain" similarly to triiodophenyl*iso*cyanate.

as determined above and the corresponding mean thickness determined from electron micrographs of sectioned sperm.

RESULTS

The normalised frequency distributions of the ratio Q for the sperm cell membrane are shown in Fig. 4, and the positions of the centre points of the main peaks are given in Table I, with estimates of the standard deviations which were found graphically on the assumption that the peaks are Gaussian in shape. It can be seen that after staining the position of the main peak undergoes a significant shift towards higher Q. Since Q represents fundamentally the ratio of the contrast of the cell membrane to that of the nucleus in the micrograph, it can be said unequivocally that the effect of the stains has been to increase the contrast of the membrane relative to that of the nucleus. Thus the differential action of the stains is established. It now remains to interpret the increases in contrast, on the basis of the theory given earlier, in order to estimate the percentage uptake by weight of the stains.

TABLE I

Positions of the Main Peaks of Fig. 4

Treatment	No. of observations	Position of peak	Standard deviation	Increase in Q
				per cent
Control	87	0.625	0.029	—
Triiodobenzoyl chloride	101	0.870	0.024	39
Triiodophenyl*iso*-cyanate	73	1.050	0.026	68

An example is given in Table II of the use of complementary measurements made with the interference microscope (I.M.) and the electron microscope (E.M.) to calculate the dry density of the sperm nucleus. (Here the "dry density" of a body means the mass of the substances it contains, other than water, divided by its total volume.) In Table III are given the densities of the nucleus, calculated in this way, for the control and samples of sperm treated with the stains. The measurements (*a*) were made within 2 days of transferring the sperm to distilled water whereas in (*b*) the sperm had been in this medium over a fortnight. The slight but systematic decrease in density from (*a*) to (*b*) is probably significant, and since the conditions of (*b*) are unnatural, in the sense that the sperm would not normally be in contact with water after the staining procedures, it was decided to compare only measurements (*a*). Making this comparison it is seen that there is no significant increase in the density of the nucleus after staining, and a mean of the three values is used in the following calculations. It must be remembered that values of ρ in the expression for Q are the real densities of parts of the section in the electron microscope, and hence to obtain ρ_N from the dry density of the nucleus, a correction has to be made for the amount of methacrylate incorporated in the structure during the embedding process. Isenberg (18) assumes that on embedding, the water in the cell is replaced by the embedding medium; for formalin-fixed ram sperm Davies

TABLE II

Example of Density Calculation

(Control Sample)

Mean dry mass of sperm head (I.M.)	Mean projected area (I.M.)	Mass thickness	Mean thickness of sperm nucleus (E.M.)	Dry density
$7.13 \pm 0.29 \times 10^{-12}$ gm.	$29.3 \pm 1.2\ \mu^2$	24.4×10^{-6} gm. cm.$^{-2}$	$0.234 \pm 0.010\ \mu$	1.04 ± 0.06 gm. cm.$^{-3}$

TABLE III

Calculated Dry Densities of the Nucleus (Dry Mass/Unit Volume)

Treatment	Dry density (gm./cc.)	
	(*a*)	(*b*)
Control	1.04 ± 0.06	0.97 ± 0.05
Triiodobenzoyl chloride	1.06 ± 0.06	1.01 ± 0.07
Triiodophenyl*iso*-cyanate	1.06 ± 0.05	0.97 ± 0.05

Mean of values (*a*) = 1.05 ± 0.03 gm./cc.

et al. (9) using the interference microscope have found that the fraction of the cell volume unoccupied by dry material is approximately 38 per cent. Using this value and a similar assumption to that of Isenberg, values of ρ_N and ρ_M corresponding to the peaks of the Q-distributions have been calculated and are given below.

Density of methacrylate,

$$\rho_B = 1.083 \text{ gm./cc.}$$

Density of embedded nucleus,

$$\rho_N = 1.46$$

Density of embedded membrane:

$$\begin{aligned}\rho_M &= 1.32 \text{ (control)} \\ &= 1.41 \text{ (chloride stain)} \\ &= 1.48 \text{ (}iso\text{cyanate stain).}\end{aligned}$$

The absolute values of ρ_M are probably subject to an error of ± 10 per cent, but the majority of the factors comprising this error will affect each value equally. The significance of the increase in density can be better judged from a comparison of the Q-distributions for each case.

Using the value mentioned above of the percentage of water in the cell and the mean value of the nuclear dry density given in Table III, calculation yields a value of 1.70 gm./cc. for the true density of the dry material of the nucleus.

DISCUSSION

From the results quoted above and from Fig. 4 it is seen that the stains used in this work have produced a statistically significant increase in the contrast of the sperm cell membrane. This result derives directly from the measurements on electron micrographs, and is, of course, not dependent on the assumption of a particular scattering theory. Since a random choice of sections was made from all parts of the sperm head, it is surprising that the curves in Fig. 4 do not show more evidence of inhomogeneity. The control distribution has only one significant shoulder, which appears again in the "chloride stain" curve at approximately the same position and possibly can be identified as the peak immediately to the right of the main one in the "*iso*cyanate" curve. The peak on the left in the "*iso*cyanate" curve probably corresponds to the presence of unstained material, as it falls immediately below the peak of the control distribution. Because of this inhomogeneity it seems logical to use the shift of the main peaks as a measure of staining, rather than the shift of the mean values of the distributions, although in any case the differences between mean and peak positions are not excessive. The measurements made with the interference microscope give values for the dry masses of the sperm heads which are in agreement with those of Davies *et al.* (9) who obtained a mean value of $6.9 \pm 0.2 \times 10^{-12}$ gm. for formalin-fixed sperm. It is interesting to note that the sperm nucleus showed no significant uptake of the stains, which may indicate that they do not react with desoxyribosenucleic acid. A similar reason is proposed by Gibbons and Bradfield (10) for the lack of contrast of nuclei in electron micrographs of sections of osmium-fixed locust testis. The value obtained for the density of the material of the ram sperm nucleus (1.7 gm. cc.) corresponds reasonably with the density of dry DNA (1.65 gm. cc.), which indicates that in the density calculation the value used for the percentage of water present is of the correct magnitude, and will, in turn, yield acceptable values in the calculation of ρ_N and ρ_M.

Fundamental to the comparative method used in interpretation of the increases in contrast is the assumption, made, for example, in equation (3) of the analysis, that there is no systematic difference in thickness between different parts of the section. In particular, the decrease in thickness of the embedding medium due to sublimation is neglected. Some of the work described in this paper was begun before the recent introduction of alternative embedding media to methacrylate, and since this medium is known to be unstable under intense electron-beam currents, special precautions were taken to reduce sublimation as far as possible. Thus micrographs were taken with a defocused condenser system, and a pre-specimen aperture was used in the electron microscope so that only small areas of the section were irradiated at one time. Some experiments which were made to check on the sublimation under these conditions by densitometry of successive micrographs of the same field showed, in fact, that the thickness of the section was increasing by from 12 A to 30 A per minute, presumably due to contamination. However, it is difficult to detect sublimation which occurs immediately on irradiation of the specimen, before micrographs can be taken. (It may be mentioned here that for the conditions under which the micrographs were taken, the "clearing" of the sections in the beam was not observed.)

The effect of sublimation and consequent

reduction in the section thickness of the embedding medium appears in equation (5) as an effectively lower value for ρ_B. Also from this equation it may be seen that $\partial\rho_A/\partial\rho_B = (1 - Q)$ and hence the error in the value of ρ_A caused by the assumption of uniform section thickness will be greatest for values of Q very different from unity. (At $Q = 1$, $\rho_A = \rho_C$ and the value of ρ_B has no significance.) From Table I it may be seen that the greatest error will occur for the control value of the sperm membrane density, for which $Q = 0.625$. If the thickness of the methacrylate were reduced by as much as 30 per cent the value of ρ_M would be decreased by about 9 per cent. However, it seems unlikely that such a large effect can be present since the density obtained for the membrane (1.32 gm. cc.) is a reasonable value for protein.

The uptake of stains, of the order of 10 per cent by weight, compares favourably with the value found on a macroscopic level by Bahr (2) for the uptake of osmium tetroxide by liver tissue, and, in fact, a few subsidiary measurements which were made on electron micrographs of osmium-stained sperm gave a mean value of Q for the cell membrane near to that found in the case of triiodobenzoyl chloride. Despite this uptake of stain, a cursory examination of two electron micrographs, one taken of a control and one of a stained section, would not be likely to show a great difference since the standard deviation of the control distribution (see Fig. 4), presumably due for the most part to random fluctuations in density, is of much the same order as the peak shifts produced by staining. On the other hand, the significance of the effect of the stains has been exhibited statistically by a relatively small number of measurements, and the differential nature of their action has been established. From the above it would appear that there are at least two classes of stain:

(*a*) There are stains which are used to increase the over-all contrast of thin sections in the electron microscope in an inquiry into the basic structure of the sectioned material. For this type of stain large uptakes are essential, which may be non-stoichiometric (15, 27). Specificity in this case is not necessary, and, in fact, may even be undesirable if the stain gives a "false" picture of what is present in the section. The test of such a stain is in the comparison of two electron micrographs, one taken before, and one after staining.

(*b*) There is a need for chemically specific electron stains which will give information about the chemical nature of cell structures. For a truly specific stain the detection of its presence in a particular area of the tissue is the information which is required by the cytologist. If the stain produces only a small increase in contrast, then its detection involves more than the comparison of the two micrographs mentioned in (*a*) (*i.e.*, as in the present work, some statistical analysis becomes necessary). Nevertheless, in this case the specificity of the stain remains the essential factor, and a large increase of contrast, though desirable, is of secondary consideration. For this reason the use of optical microscope stains, advocated by Isenberg (18) and Valentine (34) seems a useful avenue of research despite the probably small uptakes of stain which are involved. In the case of residue-specific stains, it would appear *a priori* that the more selective a stain is required to be between compounds of similar chemical composition, the less likely it is to show large variations in uptake, since the variations will only reflect the *differences* in composition. For such a selective stain a statistical method of detection similar to that used in this inquiry may be the only way of establishing unequivocally the area in which it is localised in the section.

Apart from considerations of electron staining, this method of measurement (*i.e.* relative contrast measurements in the electron microscope using a standard scattering object, and subsequent absolute determination of the standard density) could be used generally to find the density of cell structures which are below the limit of resolution of the light microscope. In this particular work a convenient standard was available in close proximity to the structure in question, but in less favourable cases it might be possible before sectioning to mix intimately some standard scattering objects of known density with the material to be examined. The advantages of such a comparative method are that a knowledge of the section thickness is not required and no assumptions about the value of the mass scattering cross-section are necessary, except that it be constant under the conditions of the experiment. The method can only be used in circumstances where the mechanism of image formation is known with some certainty and thus can only be applied to structures of much larger dimensions than the resolution of the electron microscope, when phase and interference effects are presumed to be negligible.

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References

1. Bahr, G. F., *Exp. Cell Research*, 1954, **7,** 457.
2. Bahr, G. F., *Exp. Cell Research*, 1955, **9,** 277.
3. Barrnett, R. J., and Palade, G. E., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 557.
4. Bibermann, L. M., Wtorow, E. J., Kowner, I. A., Ssuschkin, N. G., and Jaworskij, B. M., *Compt. rend. Acad. sc. U.R.S.S.*, 1949, **69,** 519.
5. von Borries, B., *Z. Naturforsch.*, 1949, **4a,** 51.
6. von Borries, B., and Lenz, F., *in* Electron Microscopy, (F. S. Sjöstrand and J. Rhodin, editors), Stockholm, Almqvist & Wiksell, 1957, 60.
7. von Borries, B., and Ruska, E., *Z. tech. Physik*, 1938, **19,** 402.
8. Bradfield, J. R. G., *Nature*, 1954, **173,** 184.
9. Davies, H. G., Wilkins, M. H. F., Chayen, J., and La Cour, L. F., *Quart. J. Micr. Sc.*, 1954, **95,** 271.
10. Gibbons, I. R., and Bradfield, J. R. G., *in* Electron Microscopy, (F. S. Sjöstrand and J. Rhodin, editors), Stockholm, Almqvist & Wiksell, 1957, 108.
11. Gibbons, I. R., and Bradfield, J. R. G., *Biochim. et Biophysica Acta*, 1956, **22,** 506.
12. Gibbons, I. R., and Bradfield, J. R. G., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 133.
13. Haine, M. E., and Agar, A. W., *in* Electron Microscopy, (F. S. Sjöstrand and J. Rhodin, editors), Stockholm, Almqvist & Wiksell, 1957, 64.
14. Hall, C. E., *J. Appl. Physics*, 1951, **22,** 655.
15. Hall, C. E., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 1.
16. Hall, C. E., and Inoue, T., *J. Appl. Physics*, 1957, **28,** 1346.
17. Hillier, J., and Ellis, S. G., *in* Congrés de Microscopie Electronique, Paris, Editions de la Revue d'Optique, 1952, 79.
18. Isenberg, I., *Bull. Math. Biophysics*, 1957, **19,** 279.
19. Kempf, G., and Lenz, F., *in* Electron Microscopy, (F. S. Sjöstrand and J. Rhodin, editors), Stockholm, Almqvist & Wiksell, 1957, 67.
20. Kuhn, K., Grassmann, W., and Hofmann, U., *Z. Naturforsch.*, 1958, **13b,** 154.
21. Kuhn, K., Hofmann, U., Grassmann, W., and Gebhardt, E., *Naturwissenschaften*, 1958, **45,** 521.
22. Lamb, W. G. P., Stuart-Webb, J., Bell, L. G., Bovey, R., and Danielli, J. F., *Exp. Cell Research*, 1953, **4,** 159.
23. Leisegang, S., *Z. Physick*, 1952, **132,** 183.
24. Lenz, F., *Z. Naturforsch.*, 1954, **9a,** 185.
25. Marton, L., and Schiff, L. I., *J. Appl. Physics*, 1941, **12,** 759.
26. Mudd, S., and Anderson, T. F., *J. Exp. Med.*, 1942, **76,** 103.
27. Ornstein, L., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 809.
28. Porter, K. R., Claude, A., and Fullman, E., *J. Exp. Med.*, 1945, **81,** 233.
29. Porter, K. R., and Kallman, F., *Exp. Cell Research*, 1953, **4,** 127.
30. Revel, J. P., Ito, S., and Fawcett, D. W., *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 495.
31. Sadhukhan, P., *J. Appl. Physics*, 1958, **29,** 1235.
32. Schmitt, F. O., Hall, C. E., and Jakus, M. A., *J. Appl. Physics*, 1945, **16,** 459.
33. Uyeda, R., *J. Physic. Soc. Japan*, 1955, **10,** 256.
34. Valentine, R. C., *Nature*, 1958, **181,** 832.
35. Walker, P. M. B., *Exp. Cell Research*, 1955, **8,** 567.
36. Watson, M. L., *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 475.
37. Watson, M. L., *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 727.
38. Wentzel, G., *Z. Physik*, 1927, **40,** 590.
39. Zeitler, E., and Bahr, G. F., *Exp. Cell Research*, 1957, **12,** 44.
40. Zworykin, V. K., Morton, G. A., Ramberg, E. G., Hillier, J., and Vance, A. W., Electron Optics and the Electron Microscope, New York, John Wiley & Sons, Inc., 1945, 684–686.