PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Current and Future Prospects in Electron Microscopy for Observations in Biomolecular Structure

B. M. Siegel

Phil. Trans. R. Soc. Lond. B 1971 **261**, 5-14
doi: 10.1098/rstb.1971.0032

Phil. Trans. Roy. Soc. Lond. B. **261**, 5–14 (1971) [5]
Printed in Great Britain

Current and future prospects in electron microscopy for observations in biomolecular structure

By B. M. Siegel
Department of Applied Physics, Cornell University, Ithaca, New York, U.S.A.

[Plates 1 and 2]

The factors limiting observations with the transmission electron microscope on structure and configuration of biomacromolecules are discussed and evaluated. The present limitations are set by methods of specimen preparation and mounting. The experimental instrument we have developed that will be used to extend the spatial information content obtainable from the weakly scattering biological materials is described.

An evaluation of present capabilities of electron microscopy applied to studies on molecular structure and the configuration of biomacromolecules must distinguish three aspects of the problem: (1) instrumental capabilities, (2) characteristics of the biological specimen, and (3) inherent 'noise' limitations in specimen preparation techniques. The discussion is usually directed to the instrumental capabilities with emphasis on the limiting spatial frequencies set by the transfer function of the electron microscope, but it is clearly the nature of the macromolecular specimen, the preparative techniques and the 'noise' introduced by the background structure that set the limits of our present capabilities for studying the structure and configuration of biomolecular objects.

Substrate structure

Obscuring all observations on the low contrast biological specimens of molecular dispersions is the extremely 'noisy', thin evaporated carbon film that is still the best available substrate for mounting macromolecules. These carbon films have an essentially 'white' noise spectrum of spatial frequencies, at least down to the resolution limits of the best electron microscopes. The presence of a random distribution of all spatial frequencies in carbon films is clearly indicated by the work of Thon (1966) in which he uses a diffractometer to give Fourier transforms of a series of high resolution electron micrographs recorded with different amounts of defocus. These Fourier transforms also demonstrate the transfer function of the electron microscope, showing that the bands of spatial frequencies passed with given phase relationships depend on the particular defocus setting and spherical aberration of the objective lens as predicted by theory (Hanzen & Morgenstern 1965). Perhaps most convincing to everyone are the actual high resolution, phase-contrast electron microscope images obtained of these thin carbon film substrates with their very contrasty images, showing a random distribution of all ranges of spatial separations.

Individual molecules in dispersions will have to be mounted on some type of substrate if a semblance of their native configuration is to be preserved after the drastic treatment to which biological materials are subjected in dehydration. It may be possible to develop techniques for producing strongly linked monolayer films of certain macromolecules of biological interest. Thin crystalline arrays, if they can be produced, would provide enhanced contrast and from it

structural information could be obtained, but there must be serious concern whether such specimens could sustain the radiation damage from the high electron flux required to obtain the information in the image. The rapid deterioration of the low orders in electron diffraction patterns of protein crystals raises questions as to the level at which crystalline order can be maintained in these materials.

The best hope would appear to be firmly supporting biomacromolecules on thin stable crystalline substrate films. The periodic image produced by these crystalline films could then be subtracted in the image. We, along with others (M. Beer 1970, personal communication; Kraut & Beer 1966), are attempting to grow very thin crystalline graphite platelets only a few

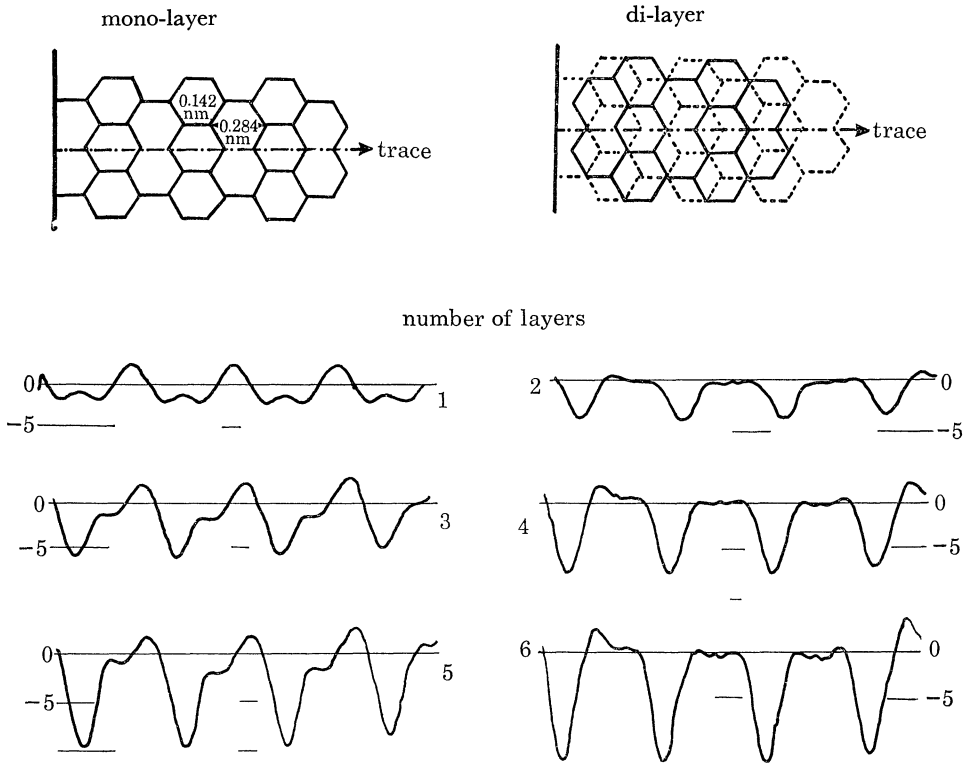


FIGURE 1. Computer traces of calculated phase-contrast images of graphite for varying numbers of layers of basal planes. The monolayer configuration of the graphite is shown on the top left and the calculated image traces for different odd numbers of layers are below it. A dilayer is shown on the right with the traces for even numbers of layers. Non-attenuated image at 170 kV; no zone plate; $C_s = 0.56$ mm.

basal planes in thickness to be used for substrates. A well-ordered graphite platelet should be stable, giving the periodic image desired and this background could be subtracted either by optical filtering such as that used so effectively by Klug and his group (Klug & DeRosier 1966) in image reconstruction or by a digital computer processing of the image. We have obtained thin graphite films supported over five micrometre holes, that show good crystalline structure in their diffraction pattern. Computer calculations on the phase-contrast images from graphite show strong 0.28 nm periodicities (Eisenhandler & Siegel 1967). In figure 1 the traces obtained in these calculations are plotted for different numbers of basal layers of the graphite. Obviously, subtraction of this periodic image could be accomplished by a digital computer or optically by filtering out the hexagonal array of sharp spots in the Fourier transform, figure 2, plate 1.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Image contrast with biomolecular specimens

Assuming we have a satisfactory periodic substrate on which biomolecular dispersions can be supported, what would be the limits set on the information that could be observed in the image after the substrate structure is completely erased? The transfer function of the electron microscope, of course, sets a limit and a great deal of attention has been given to this factor (Hanzen 1966; Hanzen & Morgenstern 1965). But the instrumental transfer function is only

Table 1. Calculated phase contrast of single atoms: effect of voltage

voltage / kV	phase contrast (%)			lens characteristics	
	carbon	nickel	gold	resolution d_0/nm	C_s†/mm
100	6.8	18.2	35.0	0.24	0.40
200	6.0	18.6	35.4	0.20	0.54
500	8.5	22.5	45.4	0.14	0.88
1000	5.3	26.8	56.0	0.11	1.30
2000	3.6	32.2	69.7	0.08	1.80
5000	0.3	36.8	87.5	0.05	3.20

$d_0 = 0.65\lambda^{\frac{3}{4}}C_s^{\frac{1}{4}}$; $\alpha_{max} = (2\Delta f/C_s)^{\frac{1}{2}}$

† C_s optimum extrapolated for super-conducting objective lens.

Table 2. Calculated phase contrast of single atoms: effect of voltage with zone plate aperture

voltage / kV	phase contrast (%)			lens characteristics	
	carbon	nickel	gold	resolution d_0/nm	C_s†/mm
100	6.2	18.0	35.0	0.18	0.40
200	6.1	17.1	31.5	0.15	0.54
500	5.9	18.7	38.2	0.11	0.88
1000	5.8	21.6	47.0	0.08	1.30
2000	5.1	24.5	55.5	0.06	1.80
5000	2.3	26.1	59.5	0.04	3.20

one of the limitations in using the transmission electron microscope for observations on biological specimens. We must also consider the relatively small scattering cross-sections of the atomic species, C, O, N, that make up biomacromolecules. These atomic scattering factors set limits on the maximum information or signal:noise ratio that can be obtained imaging these molecules even with optimum phase contrast or dark field. We (Eisenhandler & Siegel 1966*b*) have made computer calculations of phase-contrast images of single atoms considering both the transfer function at optimum phase contrast and the atomic scattering function (Burge & Smith 1962; Zeitler & Oslen 1964). The results of recent calculations on C, Ni, and Au for different voltages are given in table 1. The spatial resolutions given are those that would obtain for a 5 % difference in contrast in peak and minimum between like atoms. The advantages of a zone aperture (Hoppe 1961; Eisenhandler & Siegel 1966*a*) are shown in table 2. This table presents the contrast and spatial resolutions obtained with the same objective lens using a two-ring zone aperture defocused to optimum phase contrast. The gain is clearly the 0.05 nm increase of resolution at voltages up to 500 keV with essentially the same phase contrast.

We have simulated photographic images of planar arrays of atoms (Siegel, Eisenhandler &

Coan 1966) from our computer data that illustrate the results that could be obtained. The images obtained from a planar array of atoms is just the super-position of the single atom images of the atoms imaged in the correct x–y spatial configuration as they would project in the object plane. Figure 3, plate 1, is the simulated images of a linear chain C–C–S–S–C–C that clearly indicate the contrast to be expected. This image would be given by an electron microscope using an objective lens with a very low spherical aberration coefficient, $C_s = 0.56$ mm operated at 170 keV with a two-ring zone aperture. The image produced by an instrument operating at 500 keV without an aperture would be essentially the same as the one with the two ring zone aperture. A configuration of real biochemical interest is shown in figure 4, plate 1. Here the simulated images of two bases, guanine and cytosine labelled with methyl bromide, are shown when imaged under the same condition as the linear array in figure 3.

These simulated images represent the information that we can expect to observe by direct visual impression at the very limits of present instrumentation when imaging the primary structure of a planar molecular configuration such as the bases of a polynucleotide. These bases are in the most favourable orientation, arrayed in planar projection and 'noise' or signal from a supporting substrate is assumed to be negligible.

These 'ideal' images indicate quite clearly the limits of information on biomolecular structure that can obtain with electron microscopy at present, and at least in the immediate future. With an instrument perfected to produce images of the quality of the simulated images shown, it is a reasonable expectation that it would be possible to determine the base sequence of short sections of single strands of polynucleotides. Surface forces could well hold the planar bases parallel to the substrate and give the desired orientation. Biochemical labelling would still be required, certainly to distinguish adenine from guanine and cytosine from uracil. But we are now extrapolating to the very limits of our expectations. With the indicated capabilities it is undoubtedly more realistic to talk of observing secondary or tertiary structure of the nucleic acids, the substructure of enzymes, their bindings and interactions and the location of active sites by labelling the site with a substrate molecule containing atoms of high atomic number.

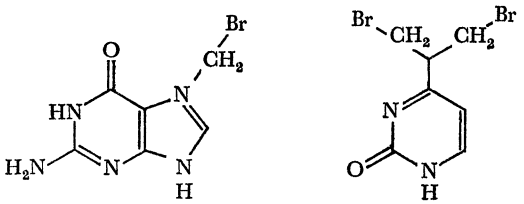
If we are to consider biomolecular configurations in which a molecule 1 to 2 nm thick is imaged with overlapping atoms in projection much more stringent conditions are required in the imaging process. The image produced has phase as well as scattering information in the

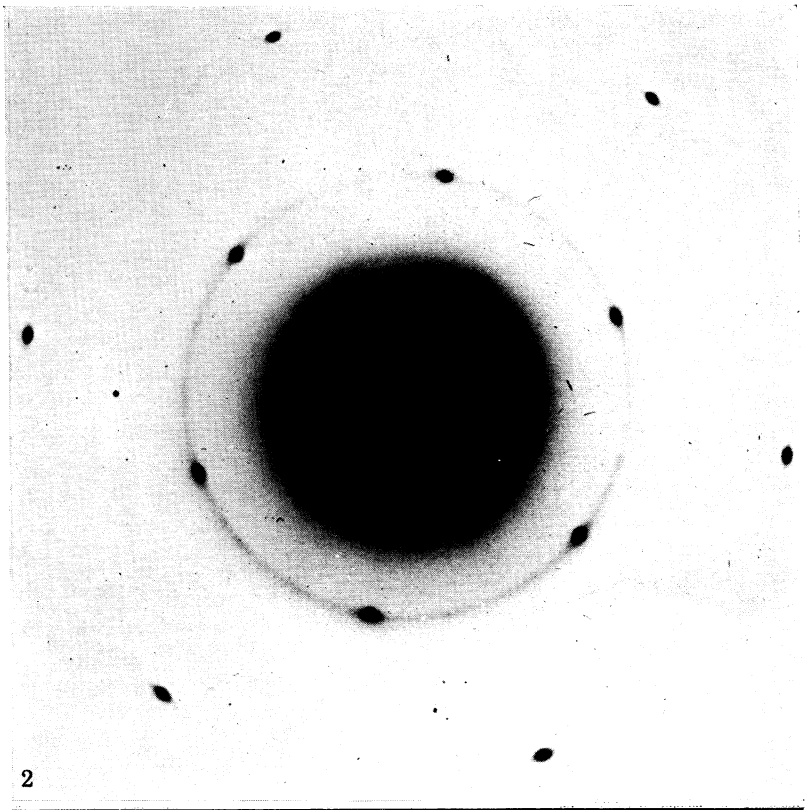
Description of plate 1

Figure 2. An electron diffraction pattern of graphite oriented with the hexagonal basal plane perpendicular to the electron beam. This pattern is the equivalent of the Fourier transform of the graphite lattice.

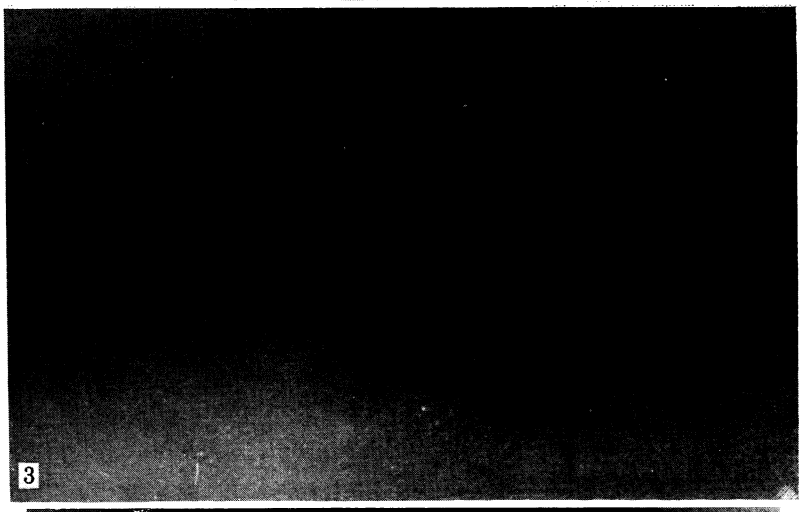
Figure 3. Simulated image of a linear atomic array C–C–S–S–C–C based on computer calculations of the phase contrast images of single atoms using an objective lens with $C_s = 0.56$ mm, 170 keV electrons, zone aperture with two rings, an dthe lens defocused to $\Delta f = 113$ nm. Essentially the same image would be obtained with 500 keV electrons, $C_s = 0.88$ mm, $\Delta f = 108.5$ nm and no zone aperture.

Figure 4. Simulated images of methyl bromine labelled guanine and cytosine using the same parameters as in figure 3.

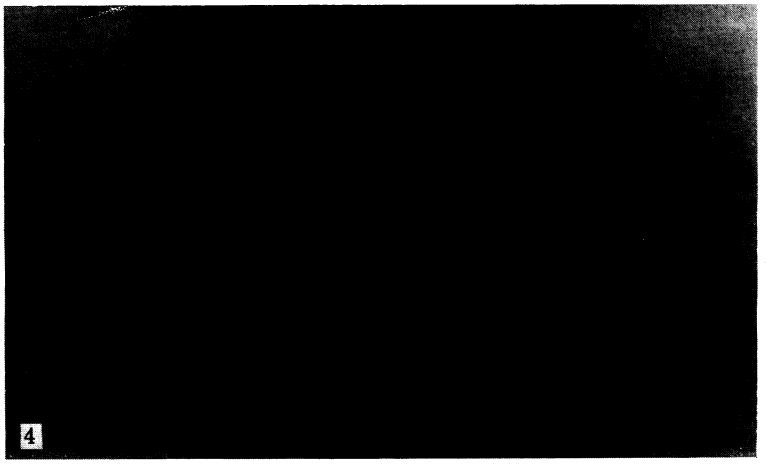




2



3



4

For legends see facing page.

Facing p. 8)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

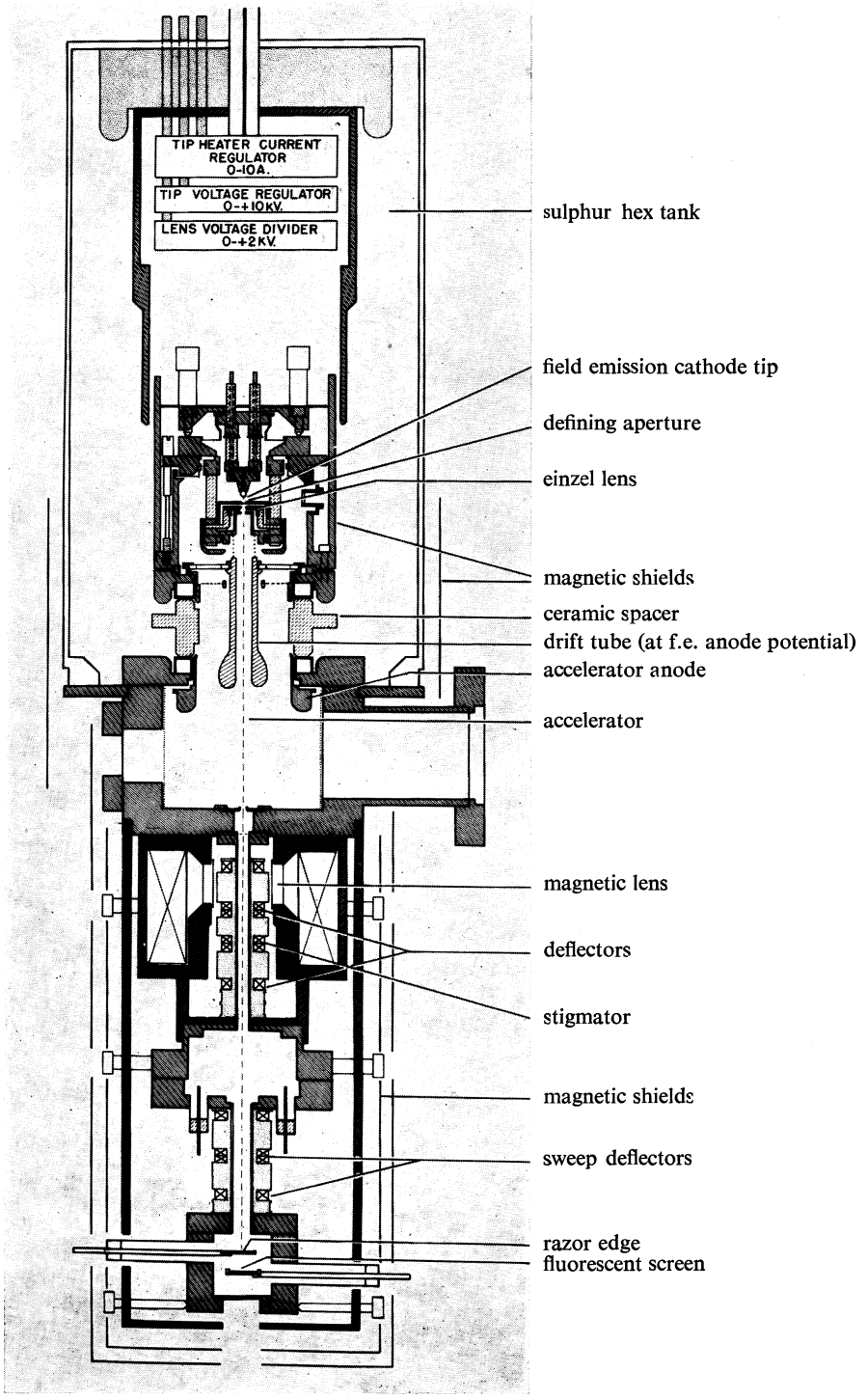


FIGURE 6. Schematic of prototype field emission gun illumination system for transmission electron microscope.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

direction of the beam and as W. Hoppe (this volume, p. 71), has suggested, it should be possible to use a computer to determine structure given this information. But as discussed later, obtaining this phase information would require recording very small contrast differences in the images and involves using very high electron fluxes through the fragile specimen. W. D. Riecke (this volume, p. 15) has proposed going to higher energies and dark field to obtain images of adequate contrast with lower radiation flux in which the separate atom image figures could be resolved even in projections of molecules of several atom layers thick.

The experimental electron microscope

Before describing the experimental electron microscope that we are developing for high resolution observations on biomacromolecules, the fundamental limits of observation set by the instrument should be reviewed briefly. The information obtained in the image is set by the transfer function (t.f.) of the electron microscope and the signal:noise ratio (S/N) at which the image can be recorded. The t.f. is determined by the spherical aberration coefficient of the objective lens, C_s, and the effective wavelength of the electrons, λ, while the ultimate S/N in the image is the statistical fluctuation in the image determined by the electron flux per image element.

We are using a superconducting lens designed for minimum C_s at 200 kV. Clearly the t.f. of the microscope could be improved by going to higher voltages, but at increased complications in the technology, and 200 kV represents an upper limit that can be used to test the performance and reliability of several innovations we are introducing. There are other ways to extend or improve the effective t.f. for example, the zone apertures (Hoppe 1961; Eisenhandler & Siegel 1966), phase plates or by filtering and phase shifting in optical reconstruction or computer processing of the image. These image processing methods are very powerful techniques and mathematical theorems (Harris 1964) indicate that in principle it should be possible to extend the spatial information obtained for the electron microscope by analytical procedures, even though the phase shifting scrambles the contrast relations in the recorded image so this information cannot be interpreted directly before image processing (Hoppe, this volume p. 71).

The other limiting factor is the signal:noise ratio available in the electron microscope image as set by the electron illumination source and the allowed exposure time for recording the image. At resolutions of atomic dimensions, i.e. 0.1 to 0.2 nm, the object elements can be only 0.025 to 0.05 nm square if a reasonable transfer function of the image shape is to be obtained. The best thermionic gun can be expected to give a maximum current density of not more than 1 A cm^{-2} at the specimen when properly apertured and collimated to give the low angular divergence and small effective size required for phase contrast, high resolution transmission microscopy. Thus at 0.1 nm resolution with object elements one-quarter that area we have only approximately 100 electrons per second per image element. With exposures of 10 s, the S/N would be 1.5:1 for an object giving 5 % contrast, and thus such low contrast detail would be below the detectable limits. If we are to extract the phase information inherent in the phase contrast image it will be necessary to record even smaller contrast differences and use computer processing to obtain this information.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Ultra high vacuum column

There are several important experimental and perhaps fundamental factors that must be considered. Perhaps most basic is radiation damage to the biological specimen that may occur at the high electron fluxes required. This is an instrumental problem only indirectly and will require careful experimental investigation under ultra high vacuum conditions that eliminate contamination and the possibility of ion damage. Only then can radiation damage caused by

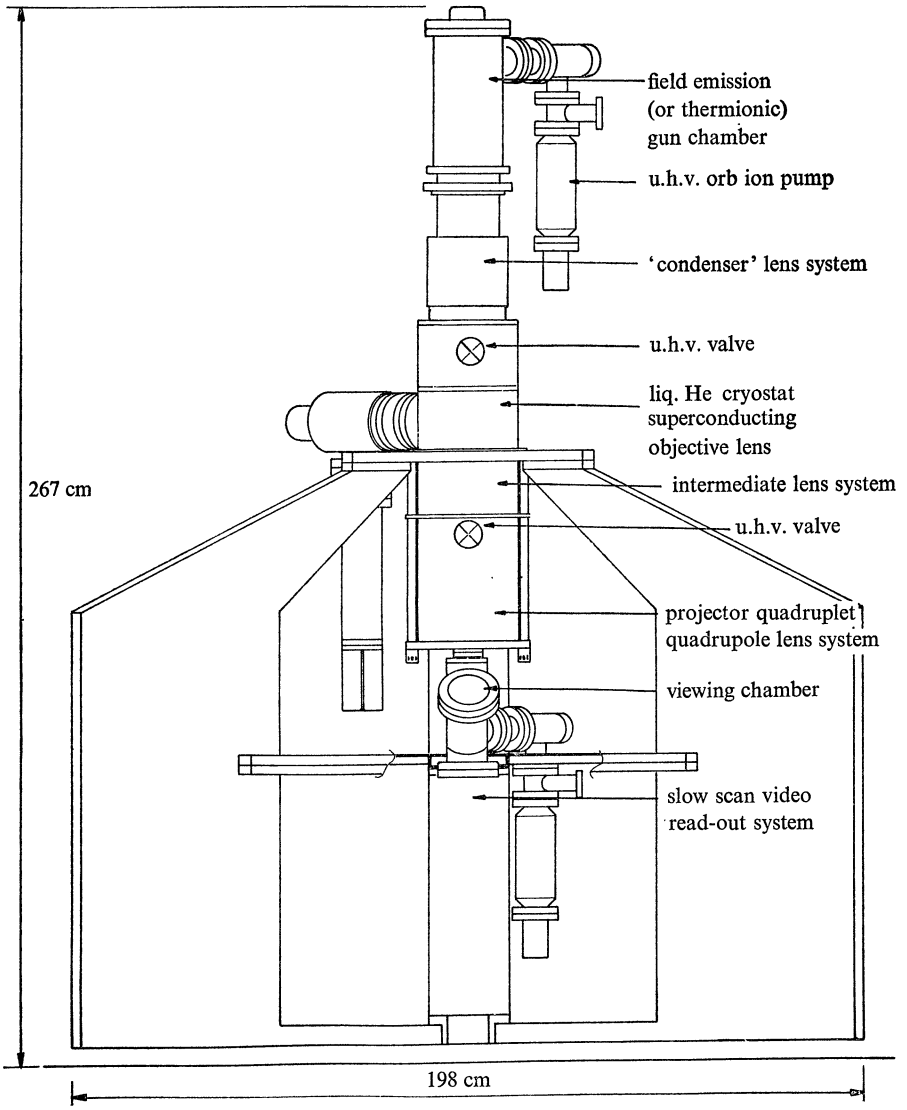


FIGURE 5. Schematic diagram of the experimental electron microscope for high resolution.

the actual electron interactions be properly investigated to determine the relative effects of electronic excitation and atomic displacements by knock-on interaction. But this problem has led us to an instrumental development that has imposed many constraints and required a great deal of attention. The instrument has been designed with a complete ultra high vacuum (u.h.v.) column. Three separate vacuum systems are being used, one each for the illuminating system, the specimen–objective lens section and the projector system. A diagram of the column is

shown in figure 5. Orb ion and sorb pumps evacuate the bakeable illuminating and projector systems and these sections are sealed off from the specimen-objective section with straight-through valves. The whole column is designed to operate at a base pressure of $\leqslant 10^{-10}$ Torr (13 nN m^{-2}) and eliminate any possible source of contamination or ions that could go directly to the specimen.

Field emission gun illuminating system

We have developed a field emission gun (Veneklasen & Siegel 1970) with an optical system designed for transmission microscopy that will change the considerations of S/N in the signal obtained over short exposure times by orders of magnitude, just as it is doing for scanning microscopy (A. Crewe, this volume, p. 61). A diagram of our prototype field emission gun

Table 3. Performance of field emission source measurements at 4 kV

beam acceptance angle, α_0/mrad	5.8
field emission current, I_0/μA	20
beam current, I_0/μA	0.01
experimental	
source size (radius/nm)	7.5 ± 2
current density, J_s/kA	9 ± 2
brightness (4 kV), β_s/MA cm^{-2} sr^{-1}	8.5 ± 2.0
brightness (projected to 100 kV)/MA cm^{-2} sr^{-1}	1700
theoretical	
brightness (4 kV), β_c/MA cm^{-2} sr^{-1}	125

and illuminating system is shown in figure 6. The field emitter tip and einzel lens inject the beam into the accelerator. Through oxygen processing of a single crystal tungsten tip oriented along the ⟨100⟩ axis enhanced emission is obtained. The measurements on this emitter system are tabulated in table 3 (Plomp, Veneklasen & Siegel 1968); the effective source size, set by the aberrations of the einzel lens, the current density and the brightness are given. At 100 kV we should have a beam of some 20 to 30 nm diameter at the specimen with a divergence of $\lesssim 10^{-4}$ rad and a current density of > 100 A/cm^{-2}. Each object point 0.05 nm square could have a flux as high as 10^4 electrons per second and using exposures of ~ 10 s, observations could be made that would record 1 % contrast differences with an S/N of 4:1. These are fundamental limits that must be brought into consideration if we are to resolve structure approaching atomic dimensions. The phase information inherent in the phase contrast image can be obtained from the weakly scattering biomacromolecules only if small contrast differences from many widely overlapping image figures can be recorded accurately.

Superconducting lens

The specimen and objective lens are contained in a special liquid helium cryostat of rigid design for high mechanical stability. This cryostat is first evacuated with sorb and orb ion pumps and then cryopumped. In addition to being an excellent cryopump, the liquid helium cryostat is, of course, needed for the superconducting lens. It also provides a number of important features for high resolution electron microscopy. The specimen is kept at liquid helium temperature and this fact may be critical in minimizing radiation damage caused by electronic excitation and charge transfer. Also only the zero point energy need be considered in the vibrational motion of the atoms, minimizing the thermal diffuse scattering of the electrons at the specimen, a factor that would degrade the image contrast, especially at higher voltages

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The whole specimen stage–objective lens system is in the cryostat at a constant temperature and thermal gradients that would introduce specimen motion are avoided.

The special superconducting lens configuration we have developed and plan to use in our experimental instrument has been described previously (Kawakatsu, Plomp & Siegel 1968). Disks of Nb_3Sn with etched annular rings are used to generate high peak fields of narrow half-width at the lens gap. A Permendur container completes the flux path and confines the fringing fields to a small axial region. A cross-section of the cryostat and lens is shown in figure 7.

Image recording

With the entire column kept under ultra high vacuum a number of constraints are introduced in the instrumental design. Perhaps the most important is the required elimination of

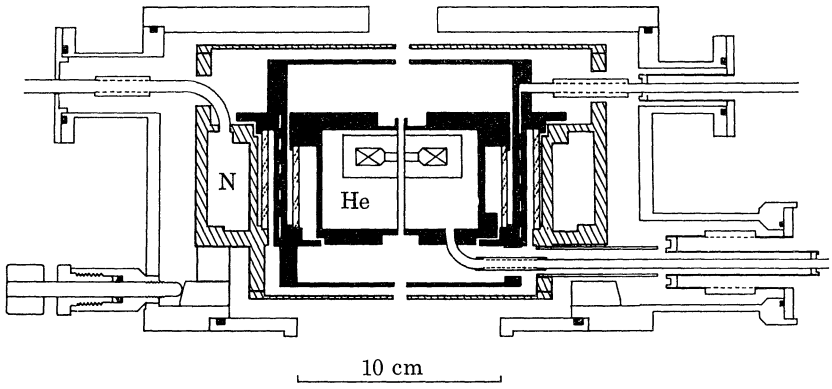


FIGURE 7. Schematic cross-section of liquid helium cryostat with superconducting lens.

photographic materials in the vacuum system. We have investigated the feasibility of placing a porous KCl target (s.e.c.) in the u.h.v. column (Siegel & Hertel 1968; Hertel 1969). Such a target would provide an effective method for directly recording the primary image without any transducer. The porous KCl target with its high resistivity and high capacitance has excellent capability for integrating over long exposure periods, set only by the capacitance limit of the target. Additional technical complications are introduced by placing the target in the column. An alternative is to use a fluorescent screen transducer, recording either by imaging the fluorescent screen on photographic film or on an external s.e.c. vidicon. The granularity in the phosphor would set a minimum size for the image element and determine the required electron optical magnification. But an analysis of the problem (Hertel 1969) indicates that with existing detectors the limits on image element dimensions are set by the effective capacitance of the detector material be it photographic film or image tubes. This capacitance is the parameter that sets the limits on what information can be stored per unit area and determines the S/N level that can be recorded without degradation in an image element of a given diameter. Hertel (1969) has made comparative evaluation of photographic media and the s.e.c. vidicon using both of these media either recording the primary image directly in the electron microscope or through a fluorescent screen transducer. The results are shown in figure 8. The abscissa gives the exposure in terms of primary electrons per square centimetre in the image plane. On the left the $(S/N)^2/cm^2$ is given for a square centimetre of detector while on the right we have the scale indicating the limiting contrast that could be recorded at an $S/N = 4$ with image elements of 400 nm × 400 nm at the detectors. An $S/N = 4$ is chosen to represent the minimum signal

to noise that can be observed with adequate reliability. (Detection reliability = 0.99, false signals = 0.05.) A perfect recording medium would have a noise figure, n.f. = 1 and give the S/N or observable contrast against exposure relation shown by the straight line bounding the curves on the left. The various curves give the exposures required to obtain a given contrast with each of the different media used, either directly or by photon coupling through a fluorescent screen. Different image element sizes would be chosen for each type of recording device to obtain the capacitance per image element required to store the necessary information. The electron optical magnification must be scaled to each device to give the required flux at the object element. Practically, these magnifications limit the size of the object field that can be imaged to fields 20 to 50 nm diameter using reasonable size recording devices.

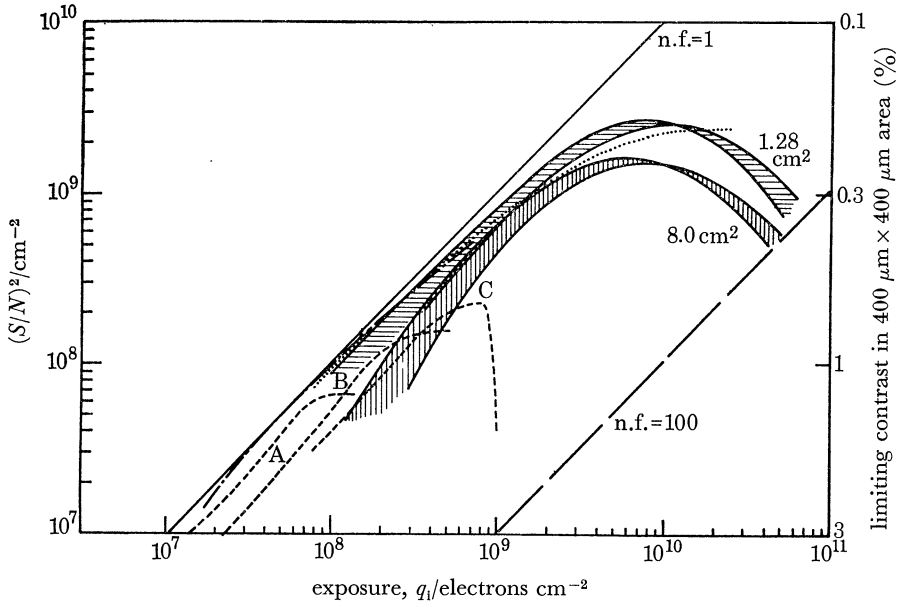


FIGURE 8. Comparative values of signal:noise ratio obtainable with photographic emulsion and the s.e.c. vidicon, both by direct recording or through a fluorescent screen transducer as a function of exposure. The $(S/N)^2$ is given for a unit area and the exposure in terms of primary electrons per square centimetre in the image. The percentages given on the right are for an $S/N = 4$ using image elements 400 μm × 400 μm. A, B and C represent different processing of photographic plates. —·—, photon coupled film; – – – –, electron image plate; . . . . ., photon coupled s.e.c.; ——, direct s.e.c.

Image processing

To fully exploit the information content of phase-contrast electron microscope images, processing of the image will be required, either by digital computer or by analog methods such as optical image reconstruction and filtering. The s.e.c. vidicon system we have developed uses a slow scan read-out of one frame per second. This system can be interfaced directly to a computer, though a disk or drum storage buffer undoubtedly would be required as a practical means of handling the huge rapid data acquisition involved. Photographically recorded images will be digitized for computer processing. Optical filtering and reconstruction is being used for rapid partial image processing, survey work and in particular for subtraction of periodic subtrate images as suggested above. Ultimately an analysis giving three-dimensional information on structure will require computer processing. But it should be repeated that the biomolecular object will have to be exposed to high electron fluxes and possible radiation damage to obtain

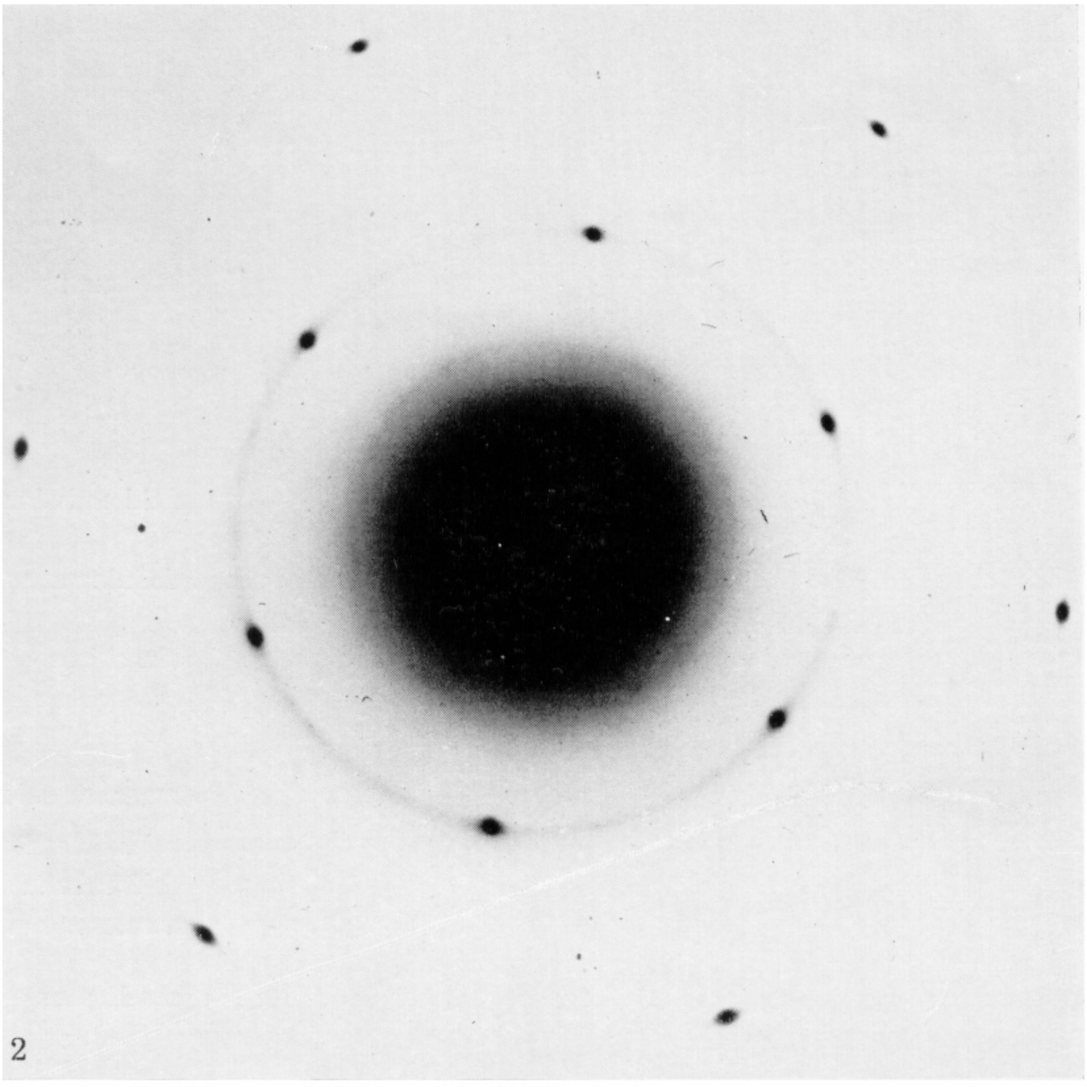
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

the requisite contrast detail for phase information and structure determinations. The prognosis on this subject is not encouraging, but it is difficult to make with any finality, and careful experimental investigations are in order.

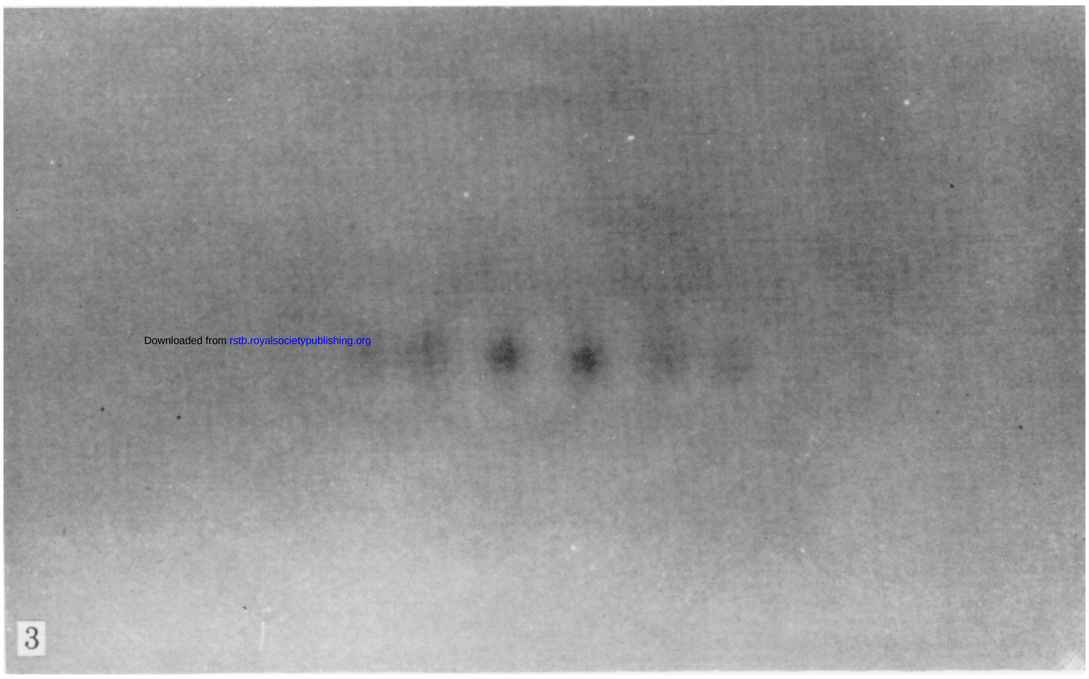
References (Siegel)

Burge, R. E. & Smith, G. H. 1962 *Proc. Phys. Soc.* **79**, 673.
Eisenhandler, C. B. & Siegel, B. M. 1966*a* *Appl. Phys. Lett.* **8**, 258.
Eisenhandler, C. B. & Siegel, B. M. 1966*b* *J. appl. Phys.* **37**, 1613.
Eisenhandler, C. B. & Siegel, B. M. 1967 *Proc. 25th Ann. Meeting EMSA*, p. 232, Baton Rouge, La. Claitor's.
Hanzen, K. J. 1966 *Proc. 6th Int. Congr. Electron Microscopy, Kyoto* 1966, **1**, 39. Tokyo: Maruzen and Co.
Hanzen, K. J. & Morgenstern, B. 1965 *Z. angew. Phys.* **19**, 215.
Harris, J. L. 1964 *J. opt. Soc. Am.* **54**, 931.
Hertel, R. 1969 Masters Thesis, Cornell University.
Hoppe, W. 1961 *Naturwissenschaften* **48**, 736.
Kawakatsu, H., Plomp, F. & Siegel, B. M. 1968 *Proc. Fourth Reg. European Conf. on E.M.* **1**, 193. Rome: Tipogr. Poligl. Vat.
Klug, A. & DeRosier, D. J. 1966 *Nature, Lond.* **212**, 29.
Kraut, A. E. & Beer, M. 1966 *J. appl. Phys.* **37**, 2179.
Plomp, F., Veneklasen, L. & Siegel, B. M. 1968 *Proc. Fourth Reg. European Conf. on E.M.* **1**, 141. Rome: Tipogr. Poligl. Vat.
Siegel, B. M., Eisenhandler, C. B. & Coan, M. 1966 *Proc. 6th Int. Congr. Electron Microscopy, Kyoto* **1**, 1966, 41. Tokyo: Maruzen and Co.
Siegel, B. M. & Hertel, R. 1968 *Proc. Fourth Reg. European Conf. on E.M.* **1**, 219. Rome: Tipogr. Poligl. Vat.
Thon, F. 1966 *Z. Naturf.* **21***a*, 476.
Veneklasen, L. & Siegel, B. M. 1970 *Proc. 7th Int. Congr. Electron Microscopy* **2**, 87. Paris: Soc. Franc. de Micros. Elect.
Zeitler, E. & Olsen, H. 1964 *Phys. Rev.* **136**, 1540.

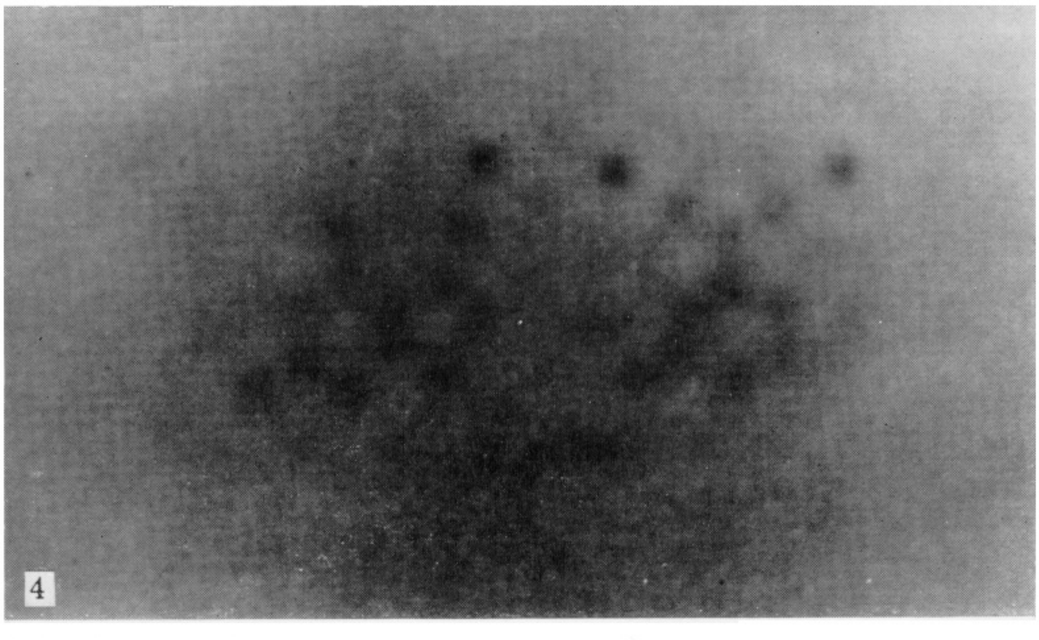
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



2



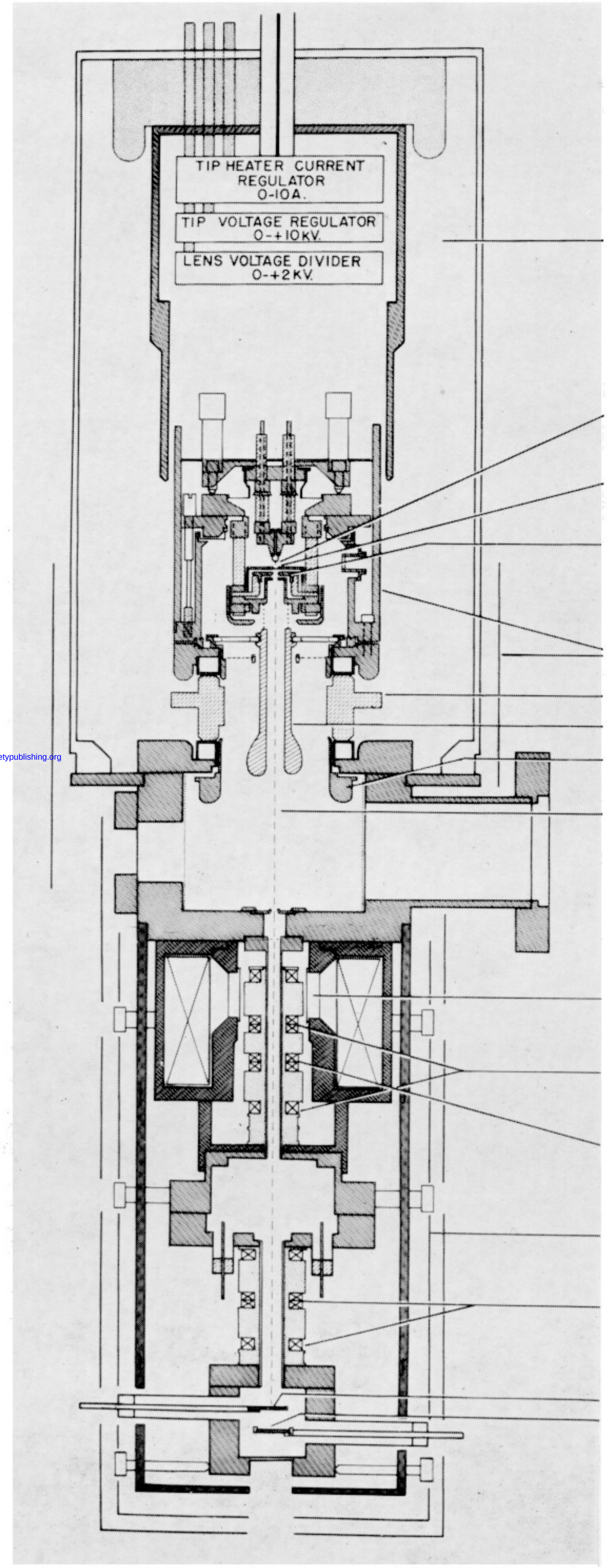
3



4

For legends see facing page.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

GURE 6. Schematic of prototype field emission gun illumination system for transmission electron microscope.