I. THE MICROSCOPE (Continued)

I.C. CONTRAST AND IMAGE FORMATION

Resolution in electron images is normally limited by contrast, NOT by lack of instrument resolving power. Though the resolving power of the microscope is of the order 0.2-0.3 nm, the resolution that can be achieved in images of most biological specimens is typically limited to about 1-5 nm. Contrast in electron images is determined by the nature and extent of interactions between the electron beam and the specimen. Properties of both the specimen (**inherent contrast**) and of the microscope system (**instrumental contrast**) need to be considered.

In simplest terms, contrast is defined as the relative difference in intensity between an image point and its surroundings.

$$Percent\ contrast = 100 \times \frac{|I_i - I_b|}{I_b}$$

where I_i = intensity of the image point
I_b = intensity of the background adjacent to the image point

I.C.1. Electron Scattering

In light microscopy, **differential absorption** of light, which depends mainly on how light interacts with various chemical stains added to the specimen, results in the visible differences in various parts of the image. In the TEM, for specimens of "normal" thickness (<100-200 nm), the portion of the beam absorbed is negligible. To be absorbed, an electron must lose **all** its energy to the specimen. Those electrons that give up part of their energy are defined as **inelastically** scattered, whereas those that give up none of their energy when scattered are **elastically** scattered.

The amount of scattering that occurs at any particular specimen point is dependent on its density and overall thickness (**mass thickness = density x thickness**) and is **relatively** independent of atomic number, chemical composition, or other specimen properties. The scattering power of a particular region of a specimen is therefore simply directly proportional to the mass thickness of the region. The probability of scattering increases with increasing mass thickness. Since contrast is determined by the average atomic number of the specimen, and biological specimens consist mainly of carbon, nitrogen, oxygen, and hydrogen atoms, the weak inherent contrast of biological specimens is a limiting problem in forming electron images with sufficient contrast. **Inherent** contrast may be increased by preferentially adding materials of high atomic number during specimen preparation. A number of preparation techniques that serve to increase the mass thickness of biological specimens to increase contrast will be discussed later in § II (The Specimen).

The path of the illuminating beam of electrons is changed when traversing the specimen either through collisions or electrostatic interactions with the atomic nuclei or with electrons in the electron shells surrounding the nuclei (Fig. I.100). Beam electrons that pass outside the range of the electrostatic field of atomic nuclei and atomic electrons are not scattered. Electrons that are scattered (deflected) may be associated with no loss of energy (**elastic collision**) or a loss of energy (**inelastic collision**).

Deflection of the beam by electrostatic interactions between electrons in the beam and the massive nuclei result in negligible energy losses of the beam electrons (Fig. I.101). Interactions that produce no change in the energy (*i.e.* wavelength or velocity) of the incident electron are termed elastic collisions. Inelastic collisions occur when energy is transferred from the beam to the electrons (negligible mass) of the specimen atoms. The proportions of inelastic and elastic collisions depend on the accelerating voltage and the nature of the specimen. For example, for a 50 nm thick carbon film illuminated with 50kV electrons, 34% of the beam is undeflected, while 11% is elastically scattered and 55% is inelastically scattered.

Since matter is mainly empty space, the trajectory of an electron must pass very close to an atomic nucleus or electron before it is deflected. Scattering of the beam electrons from direct collisions with the atomic nuclei and electrons represents a negligible factor contributing to image

contrast compared with scattering due to electrostatic deflection since the probability of a direct collision is very low because nuclei and electrons have such small cross-sections.

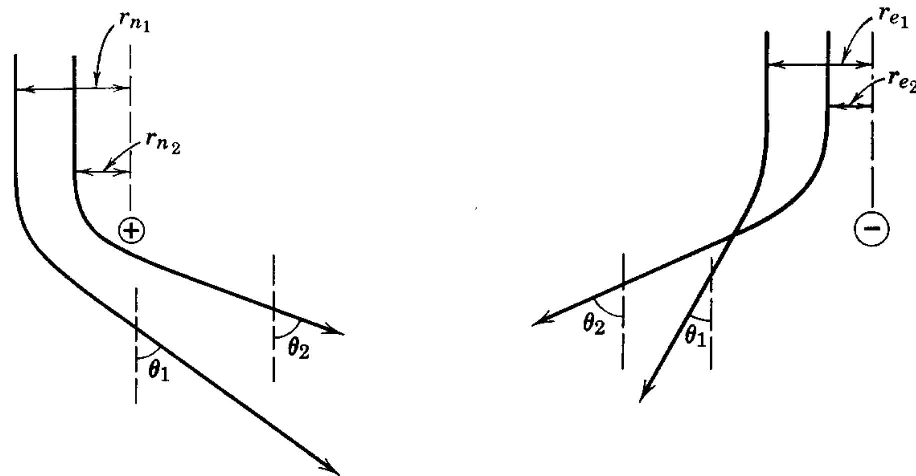

Fig. I.100. Left: Electron trajectories in the vicinity of a nucleus (elastic scattering). Right: Electron trajectories in the vicinity of a stationary electron (inelastic scattering). (From Slayter, 1970, p.423).

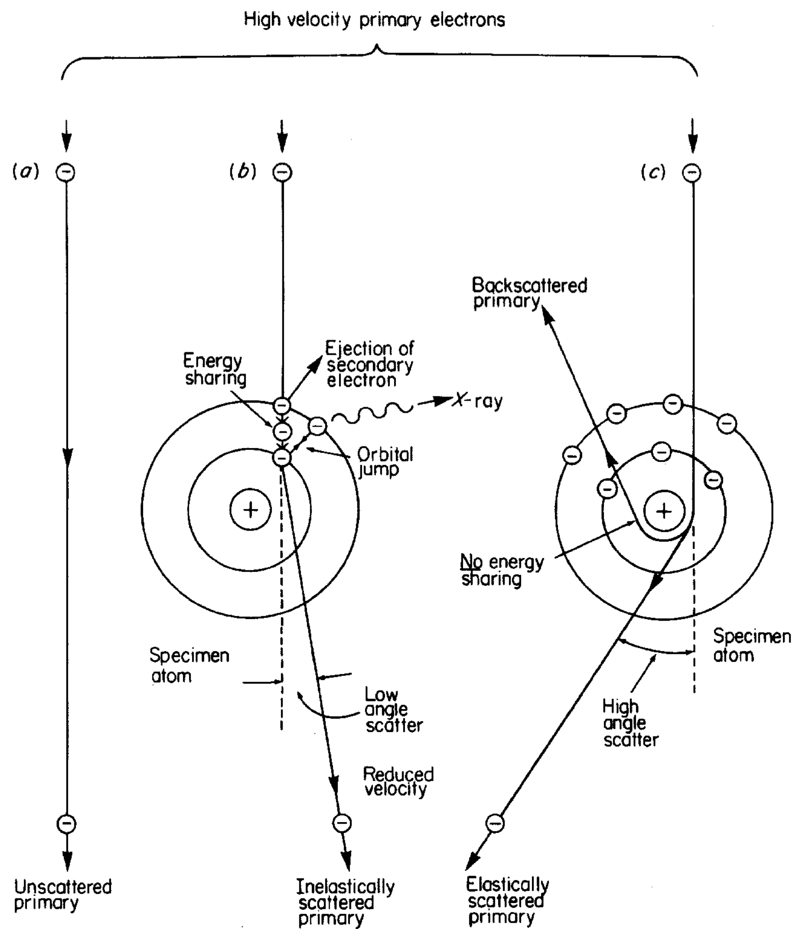

Fig. I.101. The principal modes of interaction of high-energy electrons with the atoms of matter constituting the specimen. (From Meek, 1976, p.95)

a. Elastic scattering (Figs. I.100 and I.101)

An electron passing close to a nucleus is attracted toward the positive charge of the nucleus. The electron travels a **hyperbolic path** near the nucleus and then travels a straight line, but at some angle θ with respect to the original trajectory. An electron passing closer to the nucleus is more strongly attracted by the positive charge and is therefore deflected through a larger angle. The momentum of the incident electrons is sufficient that they escape capture by the nucleus. Elastic scattering results in deflection of the incident electrons through angles up to but not much greater than 10^{-2} radians.

The angular deflection, θ_n, of the beam electron passing a distance r_n from the atomic nucleus is given by the following relationship (based on a very simplistic model for scattering):

$$\theta_n = \frac{Ze}{Vr_n}$$

where Z = the atomic number of the specimen atom
e = the charge of an electron
V = the accelerating voltage of the illumination beam (in electrostatic units)
r_n = distance of the beam electron from the stationary atomic nucleus

Thus, the deflection of the electron is directly proportional to the atomic number, Z, and decreases with distance from the nucleus, r_n, as well as with increasing beam potential, V. This expression, however, represents an oversimplification that neglects the electrostatic shielding of the nucleus by atomic electrons.

b. Inelastic scattering (Figs. I.100 and I.101)

Forces of repulsion determine electron trajectories in the vicinity of the specimen electrons. The closer the approach, the larger is the deviation of the electron trajectory. In the case of electrostatic interactions and collisions between the beam electrons and the electrons surrounding the atomic nucleus, the deflected beam electrons are likely to undergo a loss of energy (*i.e.* shift to longer wavelength and lower velocity). Scattering, in which the incident electrons lose some energy, is called **inelastic scattering.** The energy loss is generally on the order of 10-20 eV for thin specimens (<100 nm) where there is generally only one scattering event as the electron passes through the specimen. Beam electrons that lose this range of energy are deflected through very small angles (~10^{-4} radians), thus nearly all of them pass through the objective aperture.

The proportional energy loss due to each inelastic collision produces image deterioration identical to that caused if the accelerating voltage were to fluctuate on the order of one part in 10^4 or more. While the change in wavelength produced by a single electron scattering event is relatively insignificant, **multiple scattering**, which can occur in thick specimens, can be a serious source of chromatic aberration in the image.

Inelastic scattering of the beam electrons by electrostatic repulsion from the atomic electrons leads to an angular deflection, θ_e, of the electrons according to the following expression:

$$\theta_e = \frac{e}{Vr_e}$$

where r_e = distance of the beam electron from the atomic electron.

Thus, the nucleus has a higher scattering power than the atomic electron by a factor of Z, due to the greater concentration of charge in the nucleus.

Since electrons approach a "stationary" specimen atom from various directions (defined by the angular aperture of the beam), each atom behaves as a point source of scattered electrons. Thus, nuclei and individual electrons are comparable to "self luminous" points considered in light optics.

I.C.2. Amplitude/Phase Contrast

Contrast in the electron image can arise from both "amplitude" and "phase" effects. **Amplitude contrast** is produced by the loss of amplitude (*i.e.* electrons) from the beam; **Phase contrast** originates from shifts in the relative phases of the portions of the beam that contribute to the image. Unfortunately, the terms "amplitude" and "phase" contrast can be somewhat misleading, especially to those who are familiar with similar terms used in crystallography. More appropriate terms are **scattering contrast** and **interference contrast** instead of amplitude and phase contrast. However, to be consistent with most texts and articles dealing with electron microscopy, the terms "amplitude" and "phase" contrast will continued to be used in these notes. Please remember the distinction though, because this becomes important in discussing the fundamentals of image reconstruction (§ III: The Structure).

The relative importance of various contrast mechanisms is quite different in light and electron microscopy. For the majority of specimens prepared by traditional (*e.g.* staining) methods, **differential electron scattering,** an amplitude effect, is the primary source of electron contrast. The important mechanism in **light microscopy** is **differential absorption**, which is also an amplitude effect. Absorption by thin electron microscope specimens (generally < 100-200 nm thick) is negligible, whereas scattering contrast is only occasionally of importance in light microscopy. As the limit of resolution in the TEM is approached, the effects of phase contrast become more dominant. Electron phase contrast originates from two factors: 1) defocusing the image and 2) from interference between rays that are improperly focused by spherically aberrant lenses. The second mechanism is of consequence only very close to the limit of resolution.

In general, amplitude contrast is dominant for structures with large mass thickness, whereas phase contrast increases in importance for small and thin structures and becomes the dominant source of contrast for very small objects of low atomic number (*e.g.* unstained specimens).

a. Amplitude contrast

The angular distribution of scattered intensities varies as a function of the atomic composition and density of the object (Fig. I.102). **Electron opaque** object points produce appreciable scattering through relatively large angles. Thus, many of the electrons incident on such points are excluded from the lens aperture and the intensity of the images of these points is correspondingly low. Conversely, **electron transparent** regions in the object, which are of lower average atomic number and mass thickness, produce little scattering beyond the lens aperture. The intensity of images of these points is correspondingly high.

Amplitude contrast can be controlled to some extent by i) **choice of accelerating voltage** and ii) **size of objective aperture** (Figs. I.82, I.83 and I.103). Contrast is improved at lower voltages and with smaller apertures. However, unless the specimen is very thin, the higher chromatic aberration at lower accelerating voltages may lead to unacceptable loss of resolution. Gun brightness also decreases as the accelerating voltage is decreased.

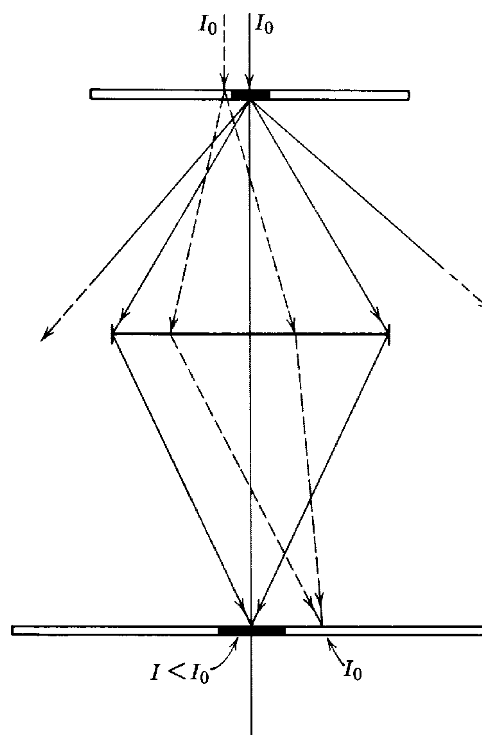

Fig. I.102. Scattering contrast in electron imaging (From Slayter, 1970, p.427)

Limiting the angular aperture of the objective lens can diminish the background intensity produced by spherical aberration. This is done with the **objective aperture** placed at the back focal plane of the objective lens (Fig. I.82). The aperture reduces the background intensity caused by spherical aberration and this enhances the contrast of image points. As the size of the objective aperture is reduced, more scattered electrons are stopped and amplitude contrast improves. If the aperture

size is made too small (<20 μm) there will be a loss of resolution due to diffraction effects (§ I.A.3.d). This may be useful though when imaging at low magnifications where resolution is not as important. A practical problem with small apertures is that they are more difficult to align in the back focal plane of the objective lens and are more susceptible to the effects of contamination, which produces lens asymmetry and thereby reduces resolution. These problems can be partially overcome with the use of a high contrast specimen holder that allows the objective lens to be operated at a longer focal length, which reduces the semi-angular aperture of the lens (Fig. I.103). However, these changes may lead to some loss of resolution owing to increased aberration effects.

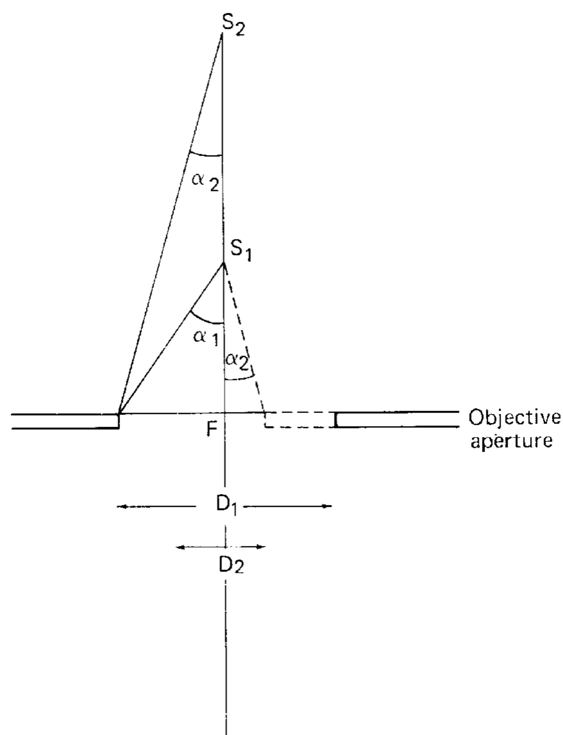

Fig. I.103. Diagram showing how amplitude contrast is increased by reduction of the effective angular aperture of the objective lens as the specimen is moved away. The dotted rays on the right-hand side of the axis show how a similar objective angular aperture may be obtained by fitting a smaller objective aperture of diameter D_2. (From Agar, 1974, p.90)

b. Phase contrast

Electron scattering can be described in terms of the diffraction of electron waves. If the interference of diffracted waves produces differences in intensity at the image, phase contrast is generated. Electrons scattered through large angles may fall outside the lens aperture, giving rise to amplitude contrast. **Ideally**, the lens focuses all other scattered electrons at the corresponding image points, where they arrive **in phase**. At planes above or below the ideal image plane, interference produces differences in intensity. These differences consist of the focus (Fresnel) fringes. Even at **EXACT FOCUS**, a slight phase contrast persists because of **spherical aberration** in the objective lens. At other focal settings, phase contrast is enhanced and produced by a combination of defocusing and lens spherical aberration.

Phase contrast in electron images arises from differences in phase between scattered and unscattered rays in different parts of the image and interference between these rays. If for the moment we neglect the effects of spherical aberration, in a fully transparent (*i.e.* no variation in refractive index or mass thickness) object, there are no phase differences and hence there will be no phase contrast in the image. **Defocusing**, in which path lengths for scattered rays are changed more than for the unscattered rays, can be used to enhance phase contrast. Contrast due to phase differences is more important than contrast due to amplitude differences for thin objects and when working near the resolution limit for the specimen.

Defocus or **phase contrast** results in the formation of strong Fresnel fringes about any part of the specimen where there is a rapid change in mass thickness (Fig. I.104). The fringes serve to enhance such points or edges by delineating them with a bright line in the under focused position (objective lens too weak) or with a dark line in the over focused position (objective lens too strong). The Fresnel fringes virtually disappear at the point of "exact" focus because this is where image contrast due to phase effects is at a minimum. Recall that, because of objective lens spherical aberration, there will ALWAYS be some fringes present even at “exact’ focus. It is common practice with thin specimens studied at high resolution to slightly under focus the objective lens to improve image contrast (Fig. I.105). At slight over focus the image is generally confusing. Initially, at slight defocus, resolution is not significantly reduced, but further defocusing reduces resolution considerably. Critical focusing is a skill learned by experience. Excessive defocusing may lead to spurious details in the image that may be erroneously interpreted as real structural details in the specimen. The defocus effect described here is an interference phenomenon similar to the effect used in phase contrast light microscopy.

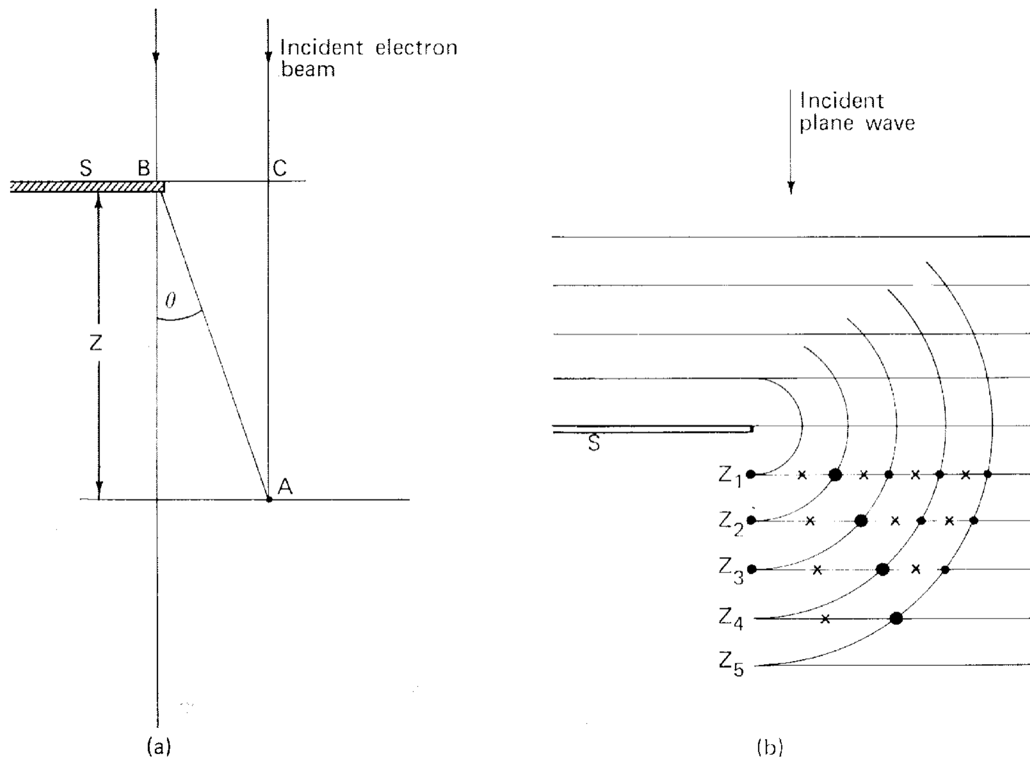

Fig. I.104. (a) A specimen S scatters the incident electron beam that interacts with the unscattered beam at a point A, distance Z behind the specimen. If this plane is viewed with the objective lens (*i.e.* the lens is over focused) a Fresnel diffraction fringe will be observed. (b) A plane wave meets a specimen S and scattered spherical waves interfere with the plane waves. Maxima of intensity are denoted by dots, and minima by crosses. (From Agar, 1974, p.97)

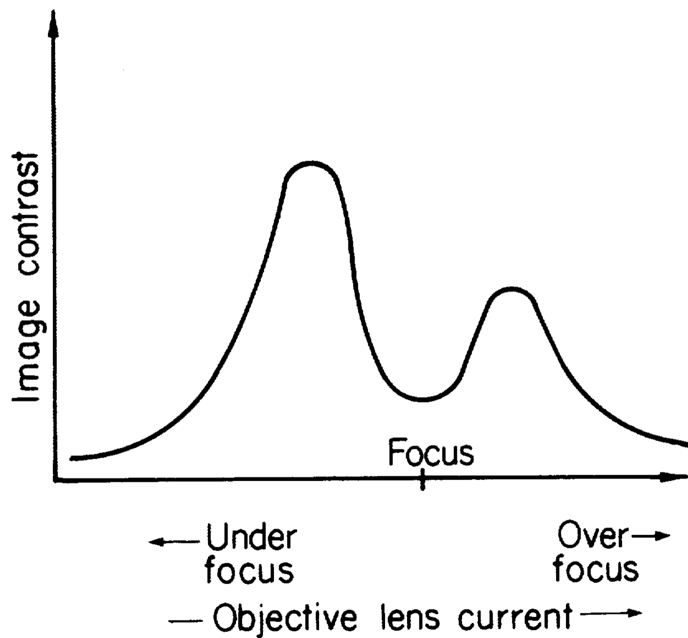

Fig. I.105. Variation of image contrast as function of objective lens focus. Fresnel fringes produce a so-called 'defocus granularity' or spurious out-of-focus contrast which is maximal at a point just below focus, where detail is enhanced by the bright under focus line. (From Meek, 1976, p.100)

I.C.3. Phase Contrast Transfer Function

The phase contrast phenomenon is responsible for the general granular background observed in pictures of carbon or other support films. The coarseness of the granularity varies with the degree of defocusing and virtually disappears at the "true" (theoretically perfect or "exact") focus setting. This granularity must be considered when interpreting high-resolution images of biological specimens since it can lead to erroneous conclusions regarding genuine fine structure in particles. The **contrast transfer function** (CTF) of the electron microscope specifies the **relative** contrast of features in the image as a function of all spatial frequencies in the image and depends both on the spherical aberration and the level of focus of the objective lens (Fig. I.106). Note that, for typical biological specimens, "optimum focus" is actually at a point where the objective lens is slightly under-focused (*i.e.* longer focal length than required for an "ideal" optical system). A detailed understanding of the effects that the microscope CTF has on electron images is essential if one wishes to obtain a reliable representation of specimen three-dimensional structure using image reconstruction procedures described in § III (The Structure).

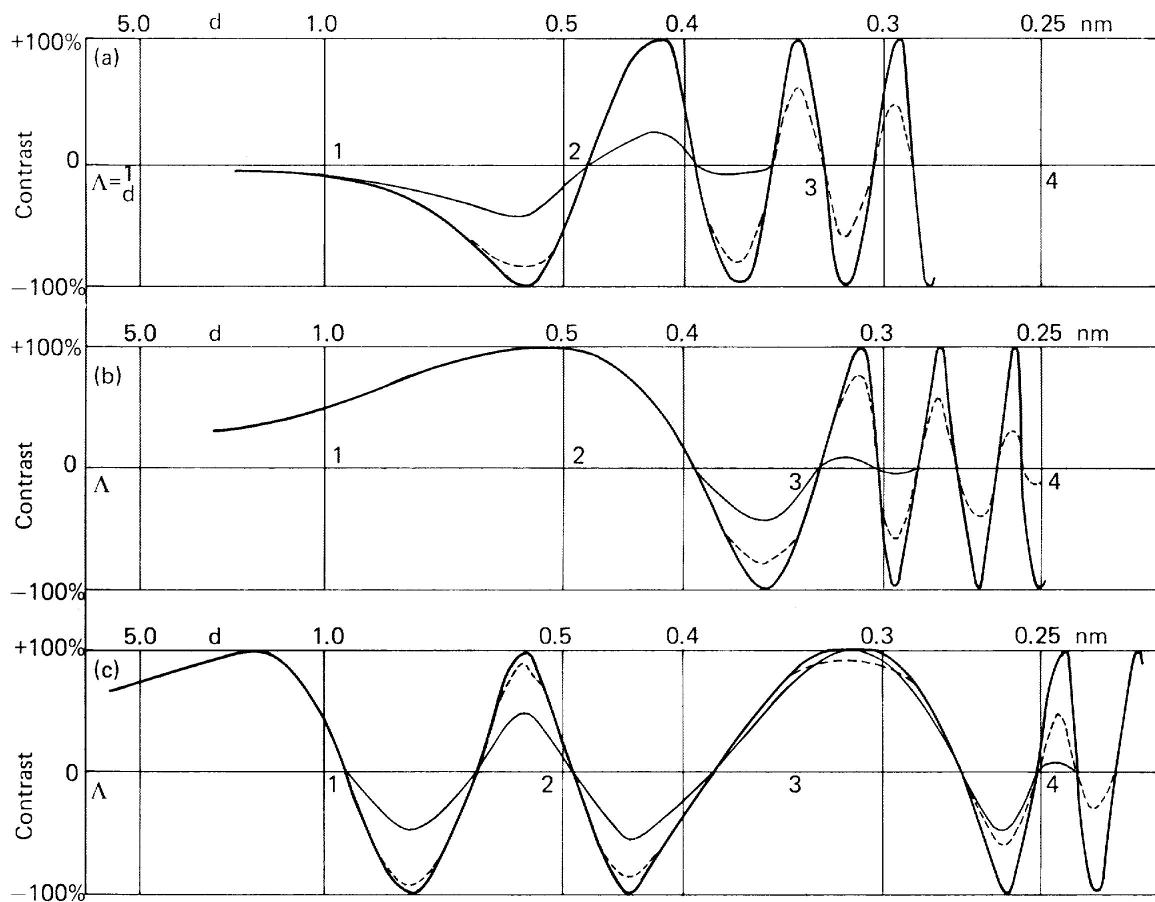

Fig. I.106. Plot of phase contrast as a function of structure size. (a) Objective lens in focus. (b) Objective lens 78 nm under focus. (c) Objective lens 234 nm under focus. (From Agar, 1974, p.282)

I.C.4. Multiple Scattering in Thick Specimens

In thick specimens, *i.e.* those generally >>100 nm, each beam electron on average suffers a number of encounters with atoms of the specimen and, hence, multiple energy losses. The energy spread in the beam emerging from the specimen may be as high as 100 eV or more and there may be relatively few or no unscattered electrons. Under these conditions there is no possibility of phase contrast from the specimen since there is no longer just one or two well-defined electron wavelengths in the beam suitable for coherent interference. Hence, contrast in images of thick specimens mainly arises from amplitude contrast.

Because of the energy losses that occur in the electron beam as it undergoes multiple inelastic interactions with the specimen, **chromatic aberration** of the objective lens will produce a limit to the resolution of the electron image according to:

$$d_{CV} = C_C \cdot \alpha_O \cdot \Delta V / V$$ (as was introduced in § I.B.3.d)

where d_{CV} = separation of two object points that are just resolved, considering voltage
C_C = chromatic aberration coefficient of the objective lens (usually 1-3 mm)
α_O = objective semi-angular aperture angle
V = accelerating potential
ΔV = maximum departure from V of electrons contributing to the image

Thus, for C_C = 2 mm, $\alpha = 5 \times 10^{-3}$ radians, ΔV = 100 volts and V = 100,000 volts:

$$d_{CV} = (2\times10^{6}\ \text{nm})(5\times10^{-3})(10^{2}\ \text{volts})/(10^{5}\ \text{volts}) = 10\ \text{nm !}$$

Since instabilities in the electrical supply to the microscope high tension are only on the order of 1-2 eV (or one part in 10^5), the thick specimen itself becomes the main limitation to the resolution obtainable in the image.

I.C.5. Other Methods for Enhancing Contrast

As was previously stated, inherent contrast in biological specimens is very weak because biological molecules primarily contain atoms of low average atomic number (*e.g.* H, C, N, O, S, P, etc.) and these all scatter electrons to about the same small extent. Contrast is therefore is generally enhanced by i) directly increasing specimen mass thickness using various preparation procedures (*i.e.* staining, shadowing, etc.) or ii) using longer exposure times in recording the photographic image. These methods are discussed in greater detail later (§ I.E.10 and § II: The Specimen).

I.C.6. References Cited in §I.C.

Agar, A. W., R. H. Alderson, and D. Chescoe (1974) Principles and Practice of Electron Microscope Operation, pp. 1-345. *In* A. M. Glauert, Ed., Practical Methods in Electron Microscopy. Vol. 2, North-Holland Pub. Co., Amsterdam.

Meek, G. A. (1976) Practical Electron Microscopy for Biologists, 2nd Ed., p. 528, John Wiley & Sons, London.

Slayter, E. M. (1970) Optical Methods in Biology, p. 757, John Wiley & Sons, New York.