II.B. RADIATION EFFECTS IN BIOLOGICAL ELECTRON MICROSCOPY

II.B.1. Introduction

Radiation damage limits the usefulness of TEM data regardless of the initial quality of the specimen. Radiation damage rather than the instrumental resolving power is the main limiting factor in obtaining high-resolution images of biological molecules.

At room temperature most biological specimens tolerate an exposure of no more than 100-500 e⁻/nm^2 (= 1-5 e⁻/Å^2). The highest resolution features of the specimen are already affected at electron exposures as low as 10 e⁻/nm^2 or less (Table 1). The structural effects observed include:

  - Mass loss as great as 50%

  - Changes in the electronic and vibrational spectra of the irradiated area, which indicate changes in the chemical bond structure at the molecular level

  - Changes in the electron diffraction pattern of the irradiated area

II.B.2. Dose/Dose Rate

Damage is proportional to the **TOTAL DOSE**, which is the product of the beam current density (dose rate) and the exposure time. For most specimens, damage is not dose-rate dependent.

Dose is a measure of the energy absorbed by the specimen. In radiology, the conventional unit is the **rad**, which is 100 ergs absorbed/gm. What is generally measured in TEM is the electron flux in coulombs/cm^2 or e⁻/nm^2 (recall that $1e^- = 1.6 \times 10^{-19}$ coulomb). Multiplication of the flux by the beam voltage gives the incident energy. However, using this quantity as a measure of the dose can be misleading, because, with a thin specimen and a fixed electron flux, the absorbed energy falls as the beam voltage rises. The conversion to rads is therefore not straightforward as it depends on the energy loss per unit length of passage through the specimen, hence on specimen atomic weight and beam voltage. For carbon and carbonaceous materials, a rough conversion from electron beam flux to radiation dose (energy) is given by 1 coulomb/m^2 (or ~6 e⁻/nm^2) at 100 kV = 4×10^7 rads. 10^9 rads is sufficient to destroy the original properties of most organic materials, whereas a dose of 10^6 rads will kill the most resistant of all living things and cause the biological inactivation of most enzymes.

The electron dose leading to complete molecular disorder in an **_unstained_** specimen is ~100 e⁻/nm^2 at 80 kV and ~250 e⁻/nm^2 at 500 kV. The minimum current density in order to barely visualize the object at the fluorescent screen is ~1 e⁻/μm^2/sec at the screen, which is a dose rate at the specimen of 400 e⁻/nm^2/sec if the magnification is 20,000X. Thus, there is not even enough time to visualize the specimen let alone focus and record an image. In a two second exposure (which would produce an optical density of about 2.0 on a photographic emulsion with a film speed of 1.0 μm^2/e⁻), the amount of energy at the specimen would be about 5×10^9 rads which is approximately equivalent to the energy delivered from a 10 megaton Hydrogen-bomb exploding 30 meters away!

II.B.3. Primary Effects of Radiation Damage to Biological Specimens

a. Introduction

Specimen damage is primarily caused by ionization resulting from the inelastic interaction of electrons with the orbital electrons of the organic material. This, in turn, leads to rearrangement of chemical bonds, formation of free radicals and diffusion of the fragments.   Whereas elastic interactions produce image contrast but no damage, inelastic interactions can produce permanent changes. The two most important structural changes that occur in biological molecules include:

  - Molecular fragmentation leading to mass loss as the material liquefies or vaporizes

  - Molecular cross-linking wherein molecules join together and molecular weight rises and the whole specimen eventually becomes one large amorphous, insoluble three-dimensional network
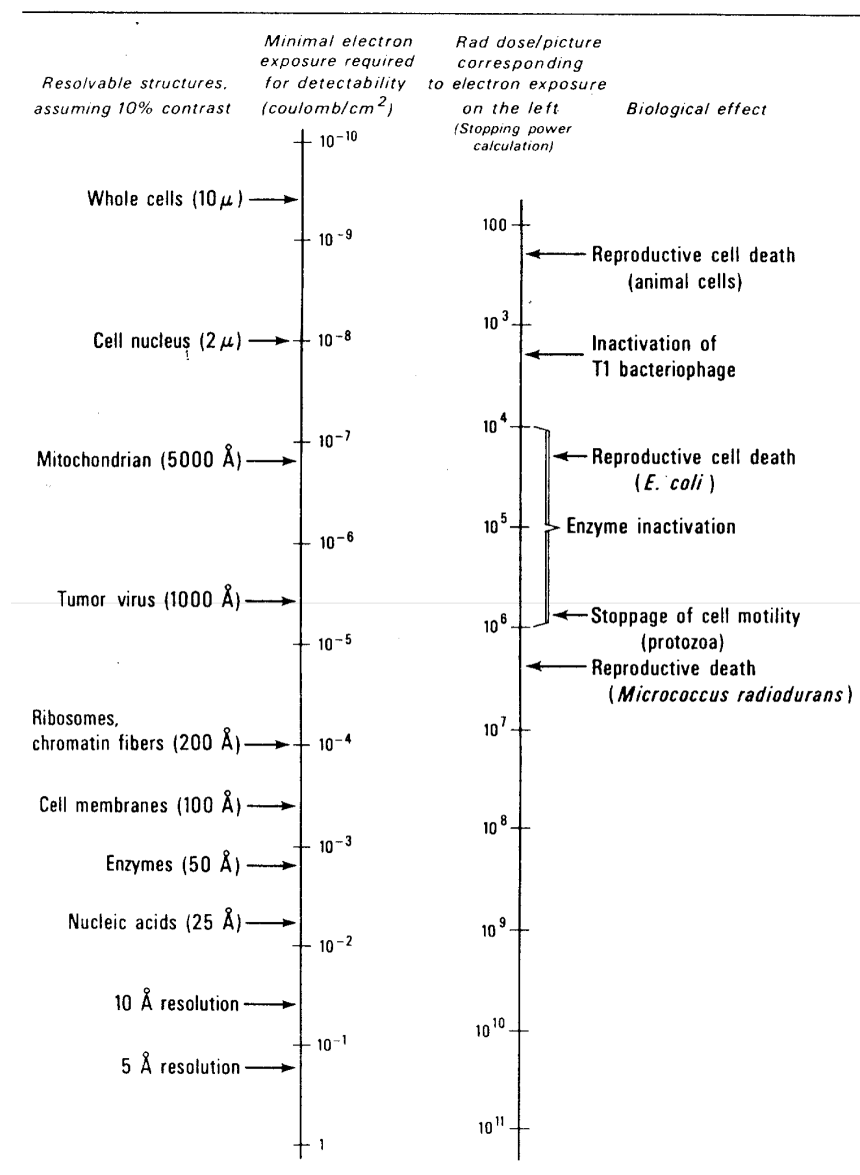
Resolvable structures, assuming 10% contrast	Minimal electron exposure required for detectability (coulomb/cm²)	Rad dose/picture corresponding to electron exposure on the left (Stopping power calculation)	Biological effect
	10^{-10}		
Whole cells (10 μ) →	10^{-9}	100	← Reproductive cell death (animal cells)
Cell nucleus (2 μ) →	10^{-8}	10^3	← Inactivation of T1 bacteriophage
Mitochondrian (5000 Å) →	10^{-7}	10^4	← Reproductive cell death (E. coli)
	10^{-6}	10^5	Enzyme inactivation
Tumor virus (1000 Å) →	10^{-5}	10^6	← Stoppage of cell motility (protozoa) ← Reproductive death (Micrococcus radiodurans)
Ribosomes, chromatin fibers (200 Å) →	10^{-4}	10^7	
Cell membranes (100 Å) →		10^8	
Enzymes (50 Å) →	10^{-3}		
Nucleic acids (25 Å) →		10^9	
	10^{-2}		
10 Å resolution →		10^{10}	
5 Å resolution →	10^{-1}		
	1	10^{11}	

Table 1. Imaging requirements and radiation damage at 1 MeV. (From Glaeser, 1975, p.215)

When an orbital electron absorbs sufficient energy to cause it to become free or reach a higher energy (non-bonding) orbital, the result is the formation of **ions** or **radicals**. Affected molecules either have a charge (ion) or an unpaired electron (radical). The fates of these species are known as the secondary events of radiation, and it is these reactions, not the primary events, that cause material damage.

b. Interactions between the electron beam and the specimen

Radiation interactions that result in bond rupture are the primary cause of damage to biomolecular structure. Specimen heating does not occur except at much higher electron intensities than those normally used. Even at higher intensities, heating would only occur subsequent to an extensive amount of direct damage.

The three primary interactions between the electron beam and the specimen are excitation, ionization, and displacement. These are all virtually temperature independent and occur very rapidly, on the order of ten femtoseconds (= 10^{-14} seconds). An excited molecule may dissipate its energy by several different routes (secondary effects), not all of which are destructive.

The excited molecule may dissipate energy by:

- **Electron, X-ray or light emission:** *e.g.* electromagnetic radiation as fluorescence or phosphorescence in the X-ray, ultraviolet or visible regions
- **Bond breakage:** chemical dissociation leads to diffusion of molecular fragments and subsequent mass loss
- **Cross-linking:** recombination of fragments can lead to identical (*i.e.* restoring the ground state molecule) or non-identical (*i.e.* new) molecular species
- **Electrostatic charging:** energy may be transferred from one molecular species to another
- **Vibrational** and **rotational excitations:** caused by damaging or non-damaging heat production
- **Chemical reactions:** reactive ions or radicals may lead to the formation of new molecules.

When beam electrons knock off electrons in the atomic orbitals of the specimen, ionization occurs and the resulting forces may cause changes in the specimen by inducing bond linkages or chemical chain scission. Bond rupture and related molecular damage associated with excitation or ionization is normally the limiting consideration in radiation damage. The cross section for atomic displacement is very much smaller than that for ionization and excitation so displacement is only expected to play a significant role in organic materials that are very resistant to damage following ionization.

c. Voltage dependence

The mechanism and amount of energy transfer from the beam to the specimen depends primarily on the voltage of the illumination beam. The <u>inelastically</u> scattered electrons, which interact with the orbital electrons of the specimen atoms and transfer large amounts of energy to them, are considered to be primarily responsible for the energy transfer that occurs from the beam. If the composition of the sample and the current density of the beam are kept constant, increasing the voltage and decreasing the sample thickness can minimize the beam-to-specimen energy transfer. At 100 keV an electron passing through a 0.1-μm thick organic specimen sustains, on average, one inelastic collision (of about 32 eV; Note: the energy of a C-C single bond is about 10 eV; 1 eV = 1.6 x 10^{-19} joule).

d. Steps in the process of beam induced damage

The major steps in the process of beam damage include:

- **Physical step**: This lasts for less than 10^{-13} sec and involves the inelastic scattering of a beam electron and results either in direct ionization or excitation of an atom. This step is virtually <u>independent of temperature</u>.
- **Physico-chemical step**: The excited atom transfers its energy by internal conversion in the molecule, leading, in a typical time of 10^{-13}-10^{-8} sec to the reintegration of the atom into the original molecule or to the formation of new molecules or radicals.
- **Chemical step**: Molecular fragments react with other molecules. This step is not limited in time.

e. Resistance to beam damage

The molecular parameter that determines a molecule's ability to resist damage by ionizing radiation is the resonance energy per π electron. The larger this quantity, the more delocalized are the π electrons and the easier it becomes for the excitation energy to be dissipated within this π electron pool without bond damage. Molecules composed of extensively conjugated, π electron systems exhibit a greater thermal stability and tend also to be less damaged by ionizing radiation. Aromatic hydrocarbons exhibit a high resistance to radiation and aromatic compounds have a protective effect on aliphatic compounds.

II.B.4. Secondary Effects of Radiation Damage

a. Nature of the damaged, beam-resistant product

Alterations that occur in the specimen take place very rapidly, so that, after just a few seconds, the specimen acquires complete morphological stability in the beam. One may conclude that, since the image does not change with time, that after a given dose, the specimen approaches a steady state with the beam, at least on a morphological level and perhaps also on a chemical level. Three experimental data corroborate this:

- **Mass loss** reaches a plateau despite continued irradiation.
- **Elemental composition** reaches a plateau after a given dose and does not change upon further irradiation.
- The **infrared absorption** pattern of an irradiated specimen becomes structureless and unchanging after a given dose.

Electron irradiation alters or destroys secondary and tertiary structure in molecules, causes loss of specific groups, increases unsaturation, causes cross-linking and/or scission of large molecules, and alters elementary composition. Cross-linking increases molecular whereas scission decreases molecular weight and produces volatile fragments. The structure of a compound dictates which of these processes dominates. The final product is determined to a certain extent by the molecular characteristics of the initial product and its molecular environment.

b. Secondary effects

Electron bombardment results in one or more of the following effects:

Chemical and physical changes	Charge effects
Mass loss	Contamination
Production of heat	Crystal structure damage

1) Chemical and physical changes

The amount of energy transferred from the beam electrons to the specimen electrons is so far in excess of chemical binding energies that no substance can be regarded as completely stable under a high voltage electron beam. Specimen stability depends on whether, after excitation of electronic energy levels, the substance falls back to its original structure or to some new configuration. Most organic materials are probably altered so as to be chemically quite different, even though the structural features within TEM resolution may not be obviously changed. The rate of chemical change can be much reduced by lowering the beam intensity to the minimum required for observation at low magnification. It sometimes helps to monitor beam induced structural changes by recording several images at time intervals.

Ionizing radiation causes a variety of molecular changes. C-H bonds are very sensitive to radiation whereas C-C bonds are more resistant. The number of C=C bonds increases with increasing irradiation dose owing to the rupture of C-H bonds and of side chains. The loss of side groups and preservation of the carbon backbone means that most organic molecules show a higher carbon content as radiation exposure is increased. The final product is predominantly, but not pure, carbon. On losing hydrogen, the organic molecule acquires more double and triple bonds. This leads to a change of bond length and angle, and thus conformational change. Amino acids appear to be most sensitive at the α-carbon where a free radical forms. Large molecules respond to irradiation with cross-link formation or scission. The ion or radical produced on a large molecule may react to form a very stable covalent bond with another radical or ion on an adjacent molecule. Such cross-linking leads to the production of large and tightly bound molecular aggregates.

2) Mass loss

All organic molecules appear to undergo mass loss upon irradiation in a vacuum. Mass loss is dependent on:
- Specimen composition

- Initial mass of the specimen
- Radiation dose and the rate (current density) at which it is delivered
- Temperature
- Vacuum conditions

Mass loss is believed to accompany the fracture or scission of the specimen molecules. Since continued scission alone would progressively reduce the mass of the specimen in the TEM, the stable product we see is either resistant to the beam or it is undergoing or has undergone cross-linking. In the TEM therefore, although specimen scission definitely occurs as seen in mass loss, the predominant reaction must be cross-linking. The rate of mass loss is initially rapid but, with continued irradiation, levels off and reaches a plateau. The variation of loss of mass with beam intensity is attributed to heat since it is believed that, if molecular fragments have a greater mobility, there is a greater opportunity for loss of substance to the vacuum. Mass loss generally occurs in the first few seconds of illumination.

3) Thermal effects

As the energy of the beam electron is reduced as it passes through and interacts inelastically with the specimen, a point is reached at which it can no longer electrically excite. At that point it imparts energy to lower energy states (*i.e.* vibrational, rotational, and translational, all of which lead to heat production). The temperature of a specimen is a function of the difference between heat input and heat dissipation. Parameters of importance include:

- Rate of energy delivery (current density and voltage)
- Thermal conductivity of the specimen
- Proximity of specimen to a good thermal conductor
- Temperature of the surroundings
- Diameter of the beam (area of specimen being irradiated or heated)
- Geometry of the mounted specimen

As more energy is delivered to the specimen per unit time, heat becomes an increasingly important factor. This occurs when the current is high, the voltage is low, and the specimen is thick and contains elements of high atomic number. Specimen temperature can reach as high as 300°C. Cooling of the specimen can be effective only if it is supported so as to ensure good thermal conductivity. The most effective way to reduce heating is to keep the current density to the minimal practical value for observation on the final screen.

It is possible that higher mass loss in heated specimens is due to greater mobility of the molecular fragments. Fragmented monomers could be cross-linked in cold specimens before leaving the object, whereas in heated specimens they would evaporate before cross-linkage could occur.

4) Charge effects

The mechanism of the effect is believed to be an uneven flow of charge onto and from the sample. During irradiation, the electrons and ions produced can lead to a radiation-induced current proportional to the radiation intensity. Charge effects probably accelerate sublimation through the ejection of small fragments as a result of electrostatic repulsion. Charge effects result mainly in the movement of particles with respect to one another or deterioration or movement of the image. The frequent practice of applying a thin layer of evaporated carbon to one or both sides of the specimen (carbon sandwich) may produce its beneficial effect by providing increased conductivity and thereby dissipates the charge.

5) Contamination

Specimens exposed to intense electron beams in a vacuum generally become coated with decomposition products of the residual vapors of oil and grease in the vacuum chamber. The principal vapors are hydrocarbons, which break down under electron bombardment, deposit on the specimen surface illuminated, and cross-link to form polymers. If these vapors are trapped away

from the specimen using an anticontaminator, water vapor becomes the chief offender. Water molecules become ionized in contact with the specimen, and carbon specimens become oxidized and etched away. The chief source of water is the photographic emulsion.

6) Crystal structure damage

Many crystalline specimens are more resistant to irradiation damage than amorphous specimens. Destruction of crystalline order is ascribed primarily to ionization and thermal effects.

Protein crystals may contain a considerable amount of water of crystallization that remains with them in vacuum at room temperature. If, however, such crystals are heated in a vacuum due to beam effects, the water of crystallization is driven out and the crystals shrink or become distorted.

II.B.5. Measuring Damage and Critical Dose

A variety of criteria have been used to measure radiation damage, including total mass loss, loss of specific elements, loss of crystalline structure, and changes in the infrared, visible, or ultraviolet spectra (including energy loss spectra). Most often, the extent of damage is assessed using radiation-induced changes in electron energy loss spectra and in electron diffraction patterns. As measured by infrared spectroscopy, the secondary structure of proteins is completely randomized after doses of 60-200 e$^-$/nm^2.

a. Electron diffraction vs. energy loss spectroscopy

Electron diffraction records primarily the loss of order in a periodic specimen. Energy loss spectroscopy is used to measure the rate of destruction of molecular organization. Decay during irradiation usually proceeds much more slowly than the fading of the diffraction pattern, indicating that intermolecular bonds are in general broken more readily than intramolecular bonds.

Low-lying (<50 eV loss) energy-loss spectra are determined by the π and σ electron energy levels of the molecule. Thus, changes in the spectra as a function of the incident electron dose can provide information about molecular structural damage (Fig. II.76). Changes in the diffraction patterns provide information about crystal structure (or weak binding force) damage (Fig. II.77).

b. Critical dose

Critical dose is defined as that dose at which the intensity of a given peak in the electron diffraction pattern falls to 1/exp (i.e. 37%) of its original value at zero dose (Fig. II.78). This is ~50-150 e$^-$/nm^2 for unstained protein crystals at room temperature. The critical exposure for organic molecules predominantly composed of saturated bonds and irradiated with 100 keV electrons, ranges between 50-500 e$^-$/nm^2. Improvement in critical exposure at 1 MeV is only about a factor of 2-3 over that found for the same material at 100 keV. Model calculations indicate that the percent reduction of intensity in diffraction patterns is roughly equal to the percent damage developed in the specimen.
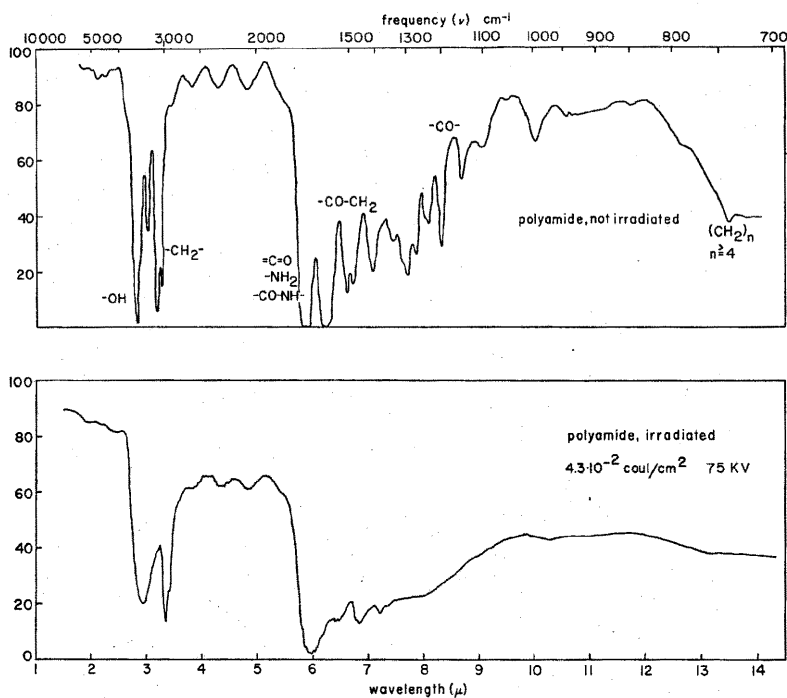

Fig. II.76. Infrared absorption spectra of non-irradiated (top) and irradiated (bottom) polyamide. (From Bahr, Johnson, and Zeitler, 1965, p.1130)
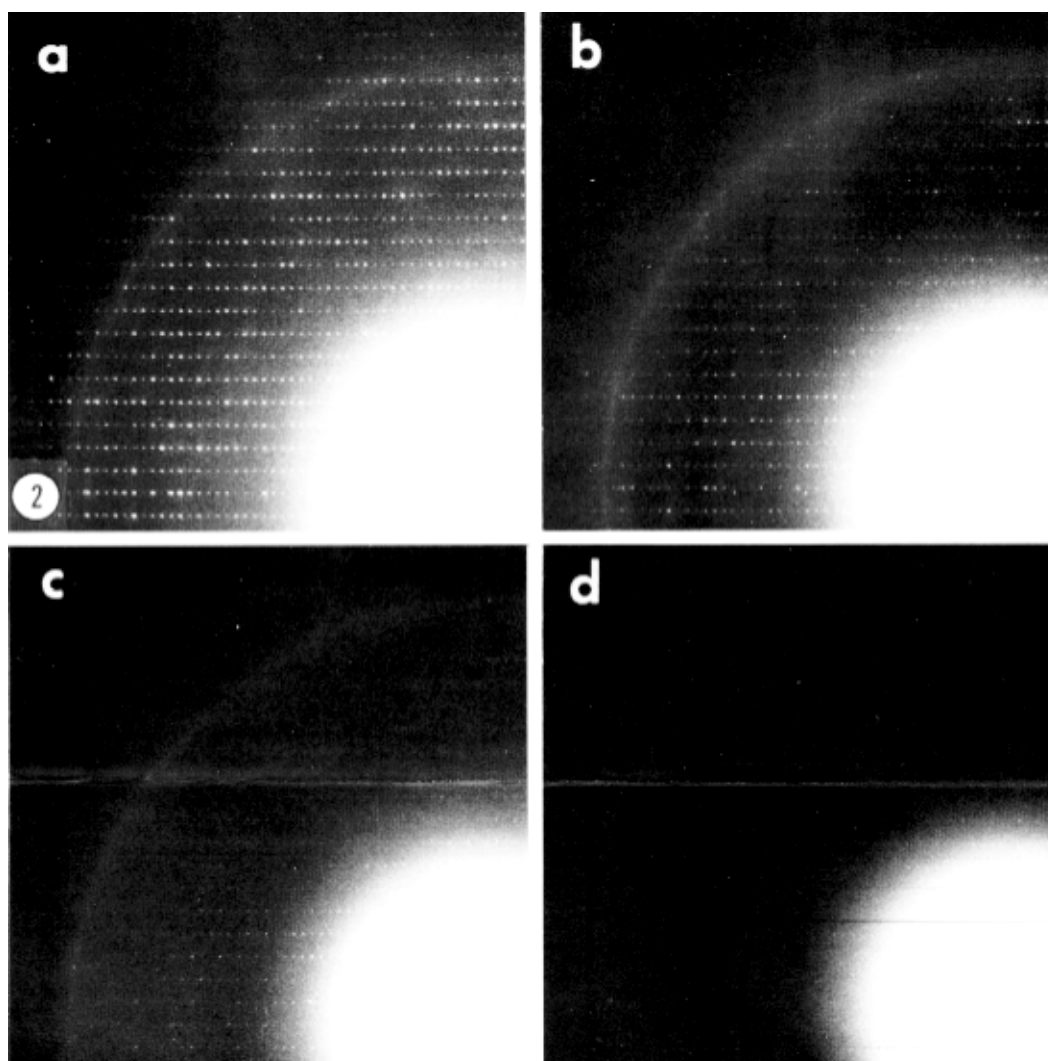
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Fig. II.77. Changes in the electron diffraction pattern of frozen-hydrated catalase crystals resulting from radiation damage. (a) The initial diffraction pattern extends to 2.8 Å. The ring present in the pattern at a spacing of 3.67 Å is arises from ice condensed on the surface of the specimen from water vapor present in the column of the microscope. (b) The pattern recorded after a dose of 2.5 e⁻/Å². (c) The pattern recorded after a dose of 5.0 e⁻/Å². (d) The pattern recorded after a dose of 11 e⁻/Å²; the pattern still extends to 8.5 Å resolution. (From Taylor and Glaeser, 1976, p.451)

The critical dose for molecular damage, as indicated by energy-loss spectra, is about ten times greater than the critical dose for crystal structure damage as indicated by the fading of the diffraction pattern.

For crystalline *l*-valine the dose for complete fading of the electron diffraction pattern is 80 e⁻/nm² at 80 kV and 200 e⁻/nm² at 500 kV. This corresponds to a dose for complete disorder of about 30 eV or one average ionizing event per unit cell. Since there are four molecules per unit cell on average, ionization of one molecule per cell is sufficient to disorder the crystalline structure. For crystalline adenosine the dose is 90 eV per molecule or three ionizing events per molecule. The greater resistance of adenosine might partially be explained by its having a conjugated π-electron system. Because of the delocalization of the outer shell valence electrons, structures of this type are relatively stable to single electron ionizations.

Radiation damage in <u>stained</u> catalase crystals is of a fundamentally different nature than for damage of <u>unstained</u> valine or adenosine crystals. The dose corresponds to about 200 ionizing events/nm² or 6000 per molecule. A significant portion of the matter within the unit cell of the

structure changes to some more stable configuration as a result of the irradiation. This would account for the changes in the relative intensities of the diffraction maxima that remain after irradiation. The distribution of matter becomes more disordered as well, since only the lower-resolution diffraction reflections remain. This redistribution of matter probably includes a significant redistribution or aggregation of stain molecules.
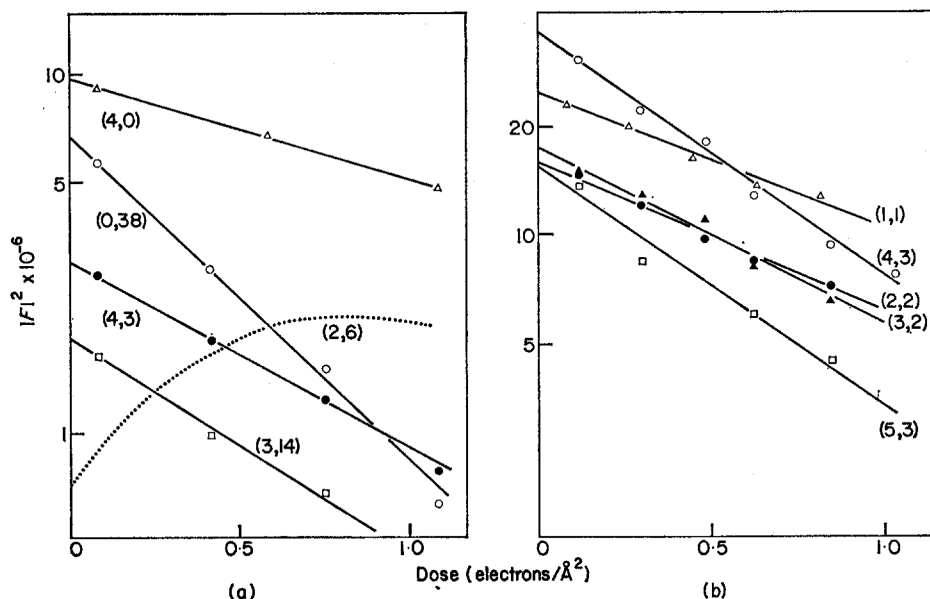

Fig. II.78. The intensities (on a logarithmic scale) of some typical reflections in (a) catalase and (b) the purple membrane electron diffraction pattern, plotted as a function of electron dose. The dotted curve is for one of the few reflections that actually increases in intensity during the initial exposure period. (Such reflections are at variance with the more typical behavior over the dose range shown, of exponential decay.) Data for (b) were taken from the "ring" diffraction patterns produced by a large number of native membranes several layers thick, so that the intensity of the (4,3) ring is contributed by both the stronger (4,3) and the weaker (3,4) lattice reflection, and so on. (From Unwin and Henderson, 1975, p.433)

c. Dose rate

Quantitative measurements of radiation damage in crystalline specimens of *l*-valine, adenosine, and uranyl-stained catalase, using fading of electron diffraction patterns, indicates there is <u>no dose rate effect</u>, such as specimen heating, in the range 0.5-50 e^-/nm^2/sec current density at the specimen.

d. Mass loss

Electron autoradiography has been used to measure the amount of mass loss in biological specimens irradiated with electrons. Loss of ^{14}C due to electron irradiation has been measured, for example, in labeled T4 bacteriophages, ribosomes, R17 virions and, *E. coli*. (Dubochet, 1975). The specific activity of labeled specimens was measured on specimens either exposed or unexposed to the beam. It was found that macromolecular biological samples are severely damaged during standard operations: from 15-40% of the mass of the sample may be lost in a 30 second exposure to the beam. During irradiation the remaining material became less and less sensitive to further carbon loss. No difference was found for positively stained verses unstained samples. A small decrease in the damage rate occurs when the beam spot size is reduced, probably leading to less specimen heating. It was found that surface migration of molecular fragments and adsorbed molecules is involved in the process of beam damage. At <u>liquid helium</u> temperature there was <u>no perceptible carbon loss</u> with irradiation. The rate and extent of mass loss with negatively stained or metal-shadowed specimens has not been reported.

II.B.6. Procedures to Reduce Radiation Damage

There are several ways to help minimize the radiation damage to biological specimens. Some of the more commonly used methods include: using fewer electrons, lower temperature, higher voltage, reducing contamination, coating specimens with a thin layer of carbon, using carbon support films, and pre-irradiating the specimen.

a. Minimize the number of electrons

The most obvious method to reduce radiation damage is to use fewer electrons either by cutting down on the exposure time or by reducing the beam intensity. A basic rule is to use the minimum magnification that will reveal detail of a given size (determined by the resolution of the photographic emulsion or CCD camera), thereby enabling the shortest possible exposure time to record the details with sufficient contrast.

A number of minimal exposure techniques have been developed for recording images of biological specimens with reduced beam damage. These techniques involve focusing on one region of the specimen but photographing another. In this way, exposure of the area of interest on the specimen to the electron beam only starts when the photograph is being taken. Initial minimal exposure techniques required the TEM grid to be translated a few microns so focusing could be performed away from the specimen. However, the translation stage movements do not remain perfectly planar to the required accuracy, hence the position of focus can change when the specimen is then translated back to the optic axis.

Modern microscopes include additional beam deflection circuitry for performing minimal irradiation procedures and allow focusing to be performed with the specimen in position so no further stage movements are necessary. The beam can be accurately aligned and deflected to a region a few μm away from the specimen. After passing through the region next to but off the specimen, the beam is re-deflected back to the optic axis so the image of the area used for focusing appears in the center of the viewing screen. When focusing and astigmatism corrections are made to satisfaction, and the exposure button is pushed, the beam is automatically recentered and expanded to a predetermined amount and an image of the desired area is recorded. After the exposure is finished, the beam is again automatically re-deflected away from the specimen to prevent further irradiation.

b. Low temperature

Chemical bonds are broken by electron impact regardless of the temperature. At low temperature some of the molecular fragments remain fixed in position and the image retains the topography of the original object after more irradiation than at room temperature, but when the specimen warms up, damage is no different than if the irradiation had occurred at room temperature. The trapping of highly reactive radicals at low temperature might also serve to reduce structural damage that would otherwise occur as a secondary effect due to chemical reactivity.

Thin crystals of the crotoxin complex (a macromolecule) embedded in glucose are preserved with a mean electron exposure about 10-fold higher at 4°K than at 300°K. These results are explained by the existence of **"caging"** effects after the specimen undergoes primary damage by ionization and excitation. "Caging" reduces the rate of migration and diffusion of the damaged fragments and also minimizes the occurrences of chemical reactions among the newly generated free radicals and ions.

The electron exposure required to completely destroy the electron diffraction pattern of frozen-hydrated catalase crystals was found to be more than ten times greater than the corresponding critical exposure found for wet hydrated catalase at room temperature. At room temperature catalase crystals lose the 6.9 nm lattice reflection in diffraction patterns after 500 e^-/nm^2. At very low temperature, the same reflection is still present after 450,000 e^-/nm^2. The lifetime of glucose-embedded catalase crystals is hardly distinguishable from that of frozen-hydrated catalase when both are observed at liquid nitrogen temperature.

Radiation damage protection found for small molecule organic compounds is generally rather limited or even non-existent at low temperatures. However, hydrated macromolecular materials show as much as a 10-fold reduction at low temperature in the rate at which radiation damage

occurs, relative to the damage rate at room temperature.

A major consequence of examining specimens at low temperature is that mass loss is greatly reduced. A dose of 1000 e⁻/nm² can result in a mass loss of 20-80% at room temperature, whereas at very low temperature, specimens are more stable and mass loss is suppressed.

c. Reduce contamination

Reduction of contamination by use of anticontaminator blades in the vicinity of the specimen helps to reduce radiation damage by removing molecules that can become ionized in the beam and subsequently attack the specimen. Hydrocarbons from the diffusion pump oil and greased gaskets generally cross-link to the specimen under the influence of the beam, thus adding mass non-specifically to the specimen and reducing its contrast.   Water molecules from insufficiently desiccated photographic films become ionized in the beam and etch the specimen (*i.e.* reduce its mass). It has been suggested that specimens placed in the microscope with the support film side facing down the column and the specimen facing the electron source helps reduce etching effects (*i.e.* mass loss) since the support film shields the specimen from water molecules entering the column from the camera chamber below.

d. Carbon stabilization of specimens and carbon support films

Coating a specimen with a thin layer of evaporated carbon has a pronounced stabilizing effect, reducing the rate of mass loss and prolonging the lifetime of diffraction patterns.

Studies have shown that crystals mounted on plastic films are more beam sensitive than crystals mounted on carbon films.   This presumably reflects the more efficient heat and electrical conductivity of carbon compared to plastic films. For the same flux, damage occurs more rapidly when a large surface of the specimen is irradiated. For thin specimens mounted on carbon films, beam damage is independent of the flux in the range 1-300 e⁻/nm²/sec. Under these conditions, specimen heating is probably negligible.

e. High voltage

The use of higher operating voltages to reduce radiation damage has been a somewhat controversial matter. While it is true that specimen lifetimes at high voltages (1 MeV) are about 2.5 times greater than at conventional voltages (50-100 keV) because faster electrons deposit less energy (*i.e.* fewer inelastic scattering events) in a specimen of given thickness, this is offset by the reduced aperture contrast at higher voltage due to fewer scattering events (elastic and inelastic) in the specimen and weaker interaction with the photographic emulsion (fewer developed silver grains, therefore less optical density and lower photographic contrast).

f. Pre-irradiation of specimens

Several studies have shown that a small dose of electrons on some specimens (primarily thin sections) helps stabilize them against further effects of irradiation. This procedure presumably works by initially favoring cross-linking reactions at low beam intensities, thus enabling specimens to withstand higher doses without mass loss due to bond scission events.

Pre-irradiation with a small dose (<100 e⁻/nm²) has been shown to be effective in reducing loss of ¹⁴C due to electron irradiation measured on labeled T4 bacteriophages and *E. coli*.

II.B.7. Relation between Contrast, Resolution and Radiation Damage

As the electron exposure is reduced, statistical fluctuations from one picture element to another can be much greater than the inherent change in image intensity from one picture element to another, *i.e.* the statistical fluctuations may greatly exceed the inherent contrast. Thus, by reducing the electron exposure, specimen detail may be well preserved, but it cannot be observed because of the noisy quality of the image. At high resolution there does not seem to be any way to escape this dilemma except through the use of image averaging techniques, which are especially powerful when applied to the study of crystalline specimens (§ III).

A fundamental limit to resolution exists because of poor statistics in the measurement. Image features with low contrast are difficult to see at low electron fluxes because the statistical

fluctuation of image intensities may exceed the small variation of intensity associated with the intrinsic contrast. The incidence of electrons at the image plane is a random process, hence the total number of electrons passing into a given image point (picture element) is n and the statistical fluctuation, or counting error is $\sqrt{n}$. For a low-contrast image feature to be resolved, the inherent contrast must exceed the statistical fluctuations by the minimum acceptable S/N ratio. Radiation damage can destroy the object to such an extent that an image with statistically well-determined contrast actually may no longer contain meaningful information about the object at the desired resolution.

II.B.8. Radiation Effects in Negatively Stained Specimens at Room Temperature

Unwin (1974) and Glaeser (1975) have studied the effects of the electron beam on negatively stained biological specimens at room and low temperatures, respectively.

Unwin analyzed stained TMV stacked disks subjected to successively higher electron doses and showed that the stain molecules migrate and redistribute themselves over the virus particle surfaces during irradiation (Figs. II.79-II.81). Morphology of the stain accessible regions of the specimen strongly influences the path of stain migration.
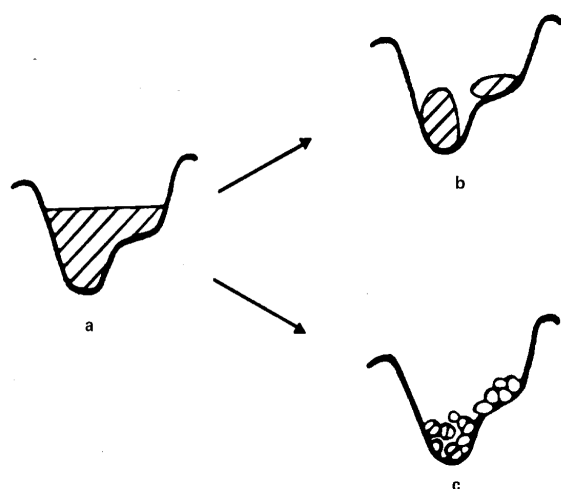

Fig. II.79. Schematic representation of the behavior of uranyl acetate (UA) and PTA under the influence of electron bombardment. Both UA and PTA scattering centers break up under the electron beam, but the UA shows more cohesion. UA is also transformed into small granular lumps. It is assumed that the specimen (thick line) is not changed under the electron beam. (a) UA or PTA stain profile before irradiation. (b) PTA stain after electron bombardment. (c) UA after electron bombardment. (From Hayat and Miller, 1990, p.16)

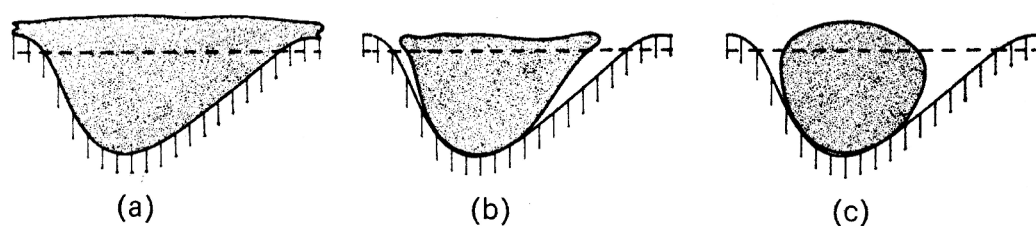(a)   (b)   (c)

Fig. II.80. Suggested influence of an asymmetrical groove in a protein molecule on the radiation changes produced in the stain near the outer surface. (a) Initial conditions, the stain near the outer surface (*i.e.* along the broken line) being centered to the right of the base of the groove; (b) the effect of contraction of the stain about its center of mass; (c) effect of surface energy included. The contraction produces a small net displacement of the stain towards the left near the outer surface, and this is greatly enlarged by the surface energy effect, causing the stain eventually to be centered directly above the base of the groove. (From Unwin, 1974, p.666)

With exposure, incident electrons reduce uranyl ions from their initial, 6^+ oxidation state to the 4^+ state. The stain shrinks quite drastically while it is irradiated (Figs. II.80 and II.81). The density of uranium dioxide crystals produced during irradiation is 11.0 g/cm³, which, when compared to the density of the acetate before irradiation (2.9 g/cm³), shows there is a considerable change in the density of the "stain" upon irradiation. It appears that the stain, before it becomes completely microcrystalline, behaves in an almost liquid-like manner. The protein appears to be morphologically quite well preserved and stable. The reason for the apparent stability of the protein seems to be that the major alterations taking place in it occur very rapidly, at doses smaller than those required for a high resolution micrograph even under low dose conditions (≤ 1000 e⁻/nm²). Also, when the

protein is being observed it has more or less reached a steady state condition, dissipating further incoming energy non-destructively.
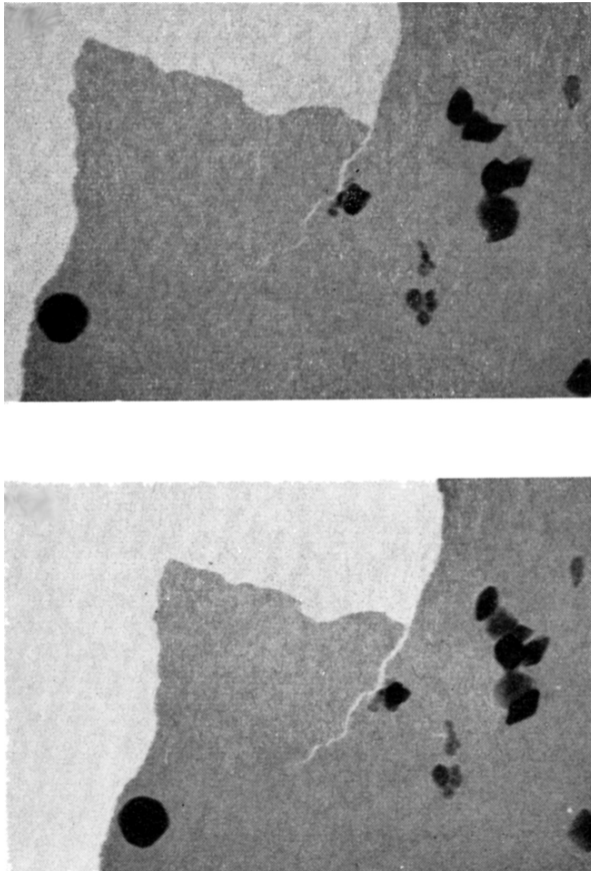

Fig. II.81. Radiation-induced changes in thin films of uranyl formate stain.   Image doses: (Top) 16 e⁻/Å². (Bottom) 625 e⁻/Å².   The images are at the same magnification and the markers on the stain film are tungsten oxide particles, which have come closer together (at bottom) as a result of its radiation-induced contraction. (From Unwin, 1974, Plate IV)
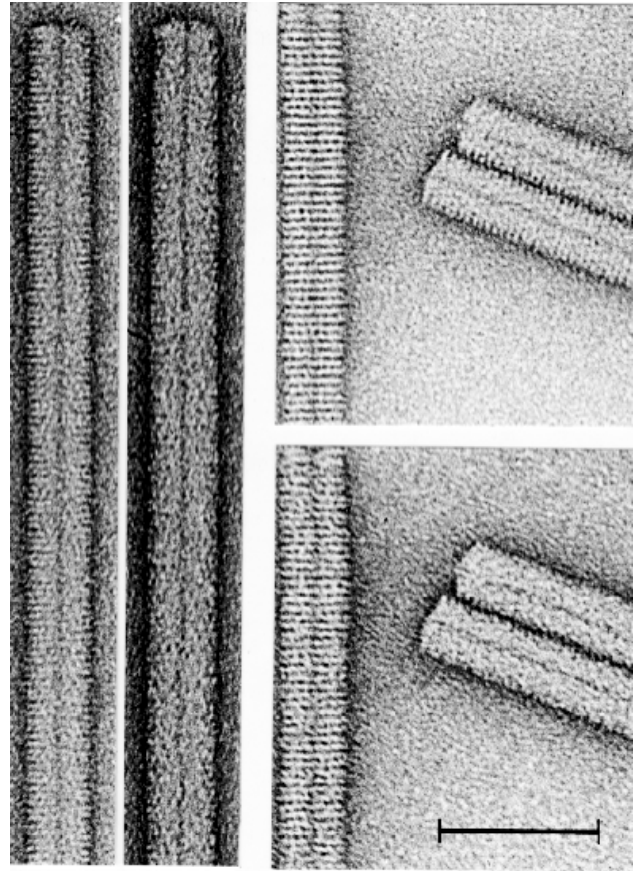
Fig. II.82. Image pairs of uranyl acetate-stained TMV, recorded with minimal irradiation methods at about 20e⁻/Å² and 200e⁻/Å², respectively. The first image in each pair clearly shows the 23 Å helical repeat of the protein subunits in the TMV rods. Finer details can also be seen in the lower-dose image of the TMV stacked disc aggregate (left edge of top and bottom right image pair). Magnification bar = 500 Å.

   The tertiary structure of **"unprotected"** protein, which is strongly dependent on hydrogen bonding as well as other weak, non-covalent interactions, is normally very rapidly disrupted by the radiation or by associated charging effects. Stain may prevent this disruption in the early stages by constraining the polypeptide chains close to their original positions and enabling extensive cross-linking to take place between them. A stable, highly cross-linked, structure would then be formed, the gross morphology of which is unlikely to be identical to that of the original protein, but at about 1 nm resolution or so should bear a close resemblance to it (but see Fig. II.82).

   There are two distinct stages of irradiation:

- An initial fixation stage (<500 e⁻/nm²) involving considerable atomic rearrangement and associated structural alterations in both stain and protein.
- A stain redistribution stage (500-50,000 e⁻/nm²) in which the protein is in a more or less stable condition but the stain continues to change.

II.B.9. Radiation Effects in Unstained, Vitrified Specimens

   This was discussed at the end of § II.A.6.c. See also Fig. II.75.

II.B.10. References Cited in §II.B.

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