DAMAGE TO BIOLOGICAL SAMPLES CAUSED BY THE ELECTRON BEAM DURING ELECTRON MICROSCOPY

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ABSTRACT A new method has been developed which measures directly the beam damage suffered by biological specimens in the electron microscope. This method involves the use of radioautography to measure specific radioactivity of labeled specimens, either exposed or unexposed to the beam. Using this technique, it has been found that macromolecular samples such as ribosomes and R17 virions are severely damaged during standard electron microscopic operations: from 15 to 40% of the mass of the sample may be lost in a 30 sec exposure to the beam.

INTRODUCTION

It is well known that biological specimens can undergo substantial alterations in appearance when exposed to the beam in an electron microscope. It seems likely that many of these alterations must be ascribed to physical or chemical damage to the specimen caused by the beam (1–8). It has been shown that many nonbiological organic materials, such as synthetic polymers and plastics, are altered chemically by relatively low doses of electrons. In particular, Reimer (9) and Bahr, Johnson, and Zeitler (10) have found that these materials actually suffer a loss of mass as a result of irradiation, under conditions thought to be comparable to those actually present in an electron microscope.

These experiments suggest that biological samples may undergo similar types of damage during microscopic examination. In the present communication we wish to describe experiments which show that this is indeed the case. Our results indicate that under normal operating conditions at high magnification, beam damage to such samples as ribosomes and virus particles is extremely high—from 15 to 40% of the hydrogen or phosphorus atoms originally present in the samples may be lost in a 30 sec exposure to the beam.

The method used to measure these losses is based upon radioautography, and is described below.

MATERIALS AND METHODS

Radioactive samples were obtained as follows. Ribosomes containing ^{3}H-labeled RNA were prepared from *Escherichia coli* cells grown on minimal medium in the presence of uridine-^{3}H; their specific activity was 75 μCi/mg of ribosomes. Ribosomes containing ^{3}H-labeled protein were the gift of Dr. Pierre Pearson (prepared from *E. coli* cells grown on ^{3}H-labeled amino acids; specific activity was 15 μCi/mg ribosomes). R17 virions containing ^{32}P-labeled RNA were the gift of Dr. Pierre Spahr (specific activity of 150 μCi/mg virions).

Oriented grids (Maxtaform, type H2, manufactured by Graticules, Ltd., London, England) were coated with a thin collodion film which was then shadowed with 100–200 A of carbon. Ribosomes or virions in solution were then adsorbed to the carbon surface by applying a 5 μl droplet (containing 0.2 mg/ml of particles in buffer A: 20 mM $Mg(OAc)_2$, 50 mM Tris-HCl buffer, pH 7.4, 100 mM NH_4OAc, 10% glycerol) to the film and allowing it to remain there from 2–4 min. Excess particles were removed by washing briefly in buffer A (less glycerol). The adsorbed particles were then stained for 6 min with 6% uranyl acetate solution, rinsed with water, and freeze-dried.

Specific squares of each grid were exposed to the electron beam according to the following protocol. Grids were placed in a Siemens Elmiskop IA (Siemens A. G., Berlin, Germany) (previously aligned and adjusted for optimum high resolution micrography using only one condenser lens) which was then brought to 40,000 ×, focused, and set at 5 μamp of beam current with the bias control. The condenser was then over-focused to provide sufficient illumination for photography with a 5 sec exposure of the film (Reproline FCK [Ansco Division, General Aniline and Film Corp., Binghamton, N. Y.], developed for 5 min at 25°C in Kodak DK-60A [Eastman Kodak Co., Rochester, N. Y.]). Without changing the illumination settings the magnification was reduced until the entire illuminated area of the specimen, or "beam spot," was contained on the final viewing screen. (When a single condenser was used the beam spot generally had a diamerer of approximately 25 μ.)

An alternative procedure was used when a double condenser system was employed. In this case the diameter of the beam spot was preset at a given value, and then the magnification raised to 40,000 × and the illumination intensity was adjusted to the appropriate level with the bias control. The magnification was then reduced until the entire beam spot was visible.

After standardizing conditions by either of these procedures, the grid was moved to a distant square, and successive areas of the entire square were exposed to the beam for the desired time.

The grid was then removed from the microscope, coated with a thin film of radioautographic emulsion (Type IL-4, Ilford Ltd., Essex, England) by the loop method of Caro, van Tubergen, and Kolb (11), and stored in the dark for 2–3 wk. After developing and fixing, each grid was returned to the electron microscope and several pictures of exposed and unexposed squares were taken (at 4000 ×). The number of silver grains in each picture was then counted, tabulated, and averaged with all other pictures from the same grid square. This averaged number of grains, referred to hereafter as grain count, was used as a measure of the radioactivity per unit area. Provided that the particles are evenly distributed over the surface of the film, the grain count is proportional to the number of radioactive atoms per particle (ribosome or virion).

RESULTS

Before this technique could be used routinely to measure the specific radioactivity of samples either exposed or unexposed to the beam, it was necessary to verify the as-

TABLE I

CONTROL EXPERIMENTS—ERROR ESTIMATES AND EFFECT OF CARBON LAYER

Number of grains per photographed area			
Normal grids		Grids coated with 200 A of carbon	
Grid 1	295	Grid 2	376
	360		402
	312		386
	333		362
	Average: 325 (±3.1%)		Average: 381 (±3.1%)
Grid 3	328	Grid 4	416
	367		388
	258		412
	275		372
	Average: 307 (±13.0%)		Average: 397 (±4.3%)
Grid 5	330	Grid 6	330
	315		446
	355		361
			462
	Average: 333 (±4.2%)		Average: 399 (±13.5%)
Grand average	322		392

Increase in grain count due to carbon: +22%.

sumption that the particles were evenly distributed over the surface of the carbon support film. That a fairly even distribution is in fact obtained was apparent when the grids were examined visually in the microscope. A quantitative proof of this point was obtained in the following way. Grids were prepared as described above, except that they were not exposed to the beam prior to radioautography. One photograph (4000 ×) from each of three or four randomly chosen squares was made, and the grains were counted and the number recorded in Table I. The average deviations of grain counts for the three (normal) grids range from 4 to 13%, indicating that the distribution of particles is fairly even and reproducible.[1]

These results indicate that the grain count is proportional to the number of radioactive atoms per particle, and hence may be used to measure any loss of material from particles caused by the electron beam. However, before this conclusion could be relied upon it was necessary to determine what effect the process of "contamination" might have on the grain count. Because of the regular use of a decontamina-

[1] The actual precision of a given measurement of beam damage is always higher than that indicated by the numbers in Table I. In a measurement, pictures from two adjacent squares (exposed and unexposed) are compared, whereas in Table I pictures were taken from widely scattered grid squares. The average deviation of numbers which determine the grain count in an actual damage experiment (usually the average of the number of grains from four pictures from a given grid square) was never higher than 6%, the average being 3.1%.

tion device the maximum thickness of the contamination layer that was deposited in 30 sec was found to be approximately 50 A (usually it was much less than this). To determine whether a layer of contamination might interfere with the radioautographic process, three grids prepared as described above were coated with approximately 200 A of carbon immediately after the radioactive sample had been applied and dried. The grain counts of random grid squares were determined, and compared with those from control grids lacking the carbon layer (Table I). The result of this quasi contamination is to increase the grain count by 22%. This is in fact the anticipated result, since the carbon layer should reduce the average energy of each β-particle passing through it, thereby increasing its probability of capture in the emulsion layer (12). (Control experiments in which the radioactivity of carbon-coated and uncoated samples was measured directly by liquid scintillation counting showed a reverse effect, namely that the carbon layer *decreased* the apparent radioactivity by 12%. This again is the result expected, the reduction being due to "quenching" by the second carbon layer.) Since the maximum observed contamination layer was 50 A, the maximum error in the grain count would be on the order of 5%. Since this error would always be positive, it could never be mistaken for a beam damage effect, but would rather lead to an underestimate of the extent of damage.

Having established the reliability of the method we proceeded to determine whether exposure to the electron beam actually results in loss of atoms from biological specimens. In the first experiment conducted, three different times of exposure to the beam were studied and the results are shown in Table II. It is clear that even a 10 sec exposure to the beam causes considerable loss of radioactivity from the sample and that the damage increases with increasing time. Thus it may be concluded that both the nature and extent of beam damage to biological specimens are extremely severe. Moreover, these results confirm earlier work done on a variety of organic and inorganic materials under simulated microscopic conditions (9, 10).

Kobayashi and Sakaoku (6) have shown that beam damage to polyethylene films

TABLE II

TIME DEPENDENCE OF BEAM DAMAGE

Time of exposure	% loss of radioactivity (damage)
sec	%
10	12
30	21
60	34

Conditions: voltage, 80 kv; sample, ^{3}H-labeled ribosomes; single condenser system.

(as measured by loss of crystallinity) decreases as the energy, or voltage, of the electrons increases. That this is also true in our system is shown in Table III. However, the fact that the extent of damage is similar at 40 and 60 kv suggests that the phenomenon of beam damage as observed by the present technique is due to a more complex set of reactions.

In all of the above experiments ribosomes carrying a tritium label in the cytosine and uracil residues were employed. It was of interest to determine whether other types of radioactive atoms in different chemical linkages were similarly affected by the beam. The behavior of phosphorus was studied by measuring the rate of loss of ^{32}P atoms from the RNA of R17 virus particles. The results are shown in Table IV, along with controls done with ^{3}H ribosomes. It is clear that ^{32}P is removed from the virus particles, and that the dependence of damage rate on voltage is similar to that seen with ^{3}H. It is not possible to make a more quantitative comparison between the rates of ^{3}H and ^{32}P loss, since the structures of the complexes in which they are bound are different. Additional experiments of this type were done in which the ^{3}H was incorporated into the ribosomal protein instead of the RNA. Identical rates of damage were observed in both cases. We conclude that beam damage as measured by the present technique is a general phenomenon, and probably affects all types of chemical groups in biological specimens.

The effect of the uranyl acetate positive stain on the damage rate was measured; no differences between stained and unstained samples were observed. We have also checked the possibility that loss in radioactivity might be due to individual whole particles falling off the grid. No decrease in the total number of particles per unit area was observed over a long period of exposure to the beam; however, the contrast between particles and background decreased significantly.

Recently, experiments have been conducted using a double condenser illumination system instead of the single condenser. No significant change in the damage rates

TABLE III

VOLTAGE DEPENDENCE OF BEAM DAMAGE

Voltage (high tension)	% loss of radioactivity (damage)
kv	%
40	37
60	37
80	24
100	15

Conditions: exposure time, 30 sec; sample, ^{3}H-labeled ribosomes; single condenser system.

TABLE IV

BEAM DAMAGE AS MEASURED BY ^{3}H AND ^{32}P

Voltage	Sample	% loss of radioactivity (damage)
kv		%
80	RNA-^{3}H in ribosomes	18
80	RNA-^{32}P in R17 virions	19
40	RNA-^{3}H in ribosomes	40
40	RNA-^{32}P in R17 virions	31

Conditions: exposure time, 30 sec; single condenser system.

were found. In addition, preliminary studies on the effect of beam spot diameter have been conducted using the double condenser system. Reduction of the beam spot diameter from 40 to 10μ produced a small decrease in damage rate (from 18 to 14%, respectively, at 80 kv, 30 sec exposure). This effect may be due to the decrease in sample temperature, which is caused by the reduction in spot diameter.

We have also made a preliminary investigation of the possibility that residual gases in the microscope column might be an important factor in the damage phenomenon, as originally suggested by Heide (5). These experiments were done in collaboration with Dr. Richard Hartmann (New York Medical College, New York) using a Hitachi HU-11 microscope (Hitachi, Ltd., Tokyo, Japan) modified for high vacuum work. At a vacuum of 3×10^{-8} Torr damage rates were observed which were closely comparable to those reported in Table III, where the vacuum was approximately 10^{-5} Torr. Thus it seems unlikely that residual column vapors are entirely responsible for the damage process, although a secondary involvement cannot be ruled out. It will be of interest to see if further reduction of the column pressure diminishes the damage rate.

DISCUSSION

The most important conclusion to be drawn from this work is that beam damage to macromolecular specimens under standard operating conditions is more severe than has been generally recognized, although quite comparable to the effects observed by others in related systems (9, 10). Thus in the case of positively stained or unstained samples it is safe to assume that from 15 to 40% of the mass of the sample will be removed during the process of focusing the microscope and making photographs. It is questionable whether the fine structure of the remaining residue bears a significant relationship to that of the original particle.

At present the chemical nature of the reactions leading to the observed loss of mass is not known, although the over-all process is probably similar to that described by Reimer (9). According to this model, ionization of the specimen is caused

by collision of beam electrons with specimen electrons, and this leads to cleavage of covalent bonds and the formation of low molecular weight degradation product. As the mass of these degradation products becomes smaller and smaller due to continued bond cleavage events, they may be expected simply to evaporate, thereby reducing the mass of the parent macromolecule. However, the observed kinetics of the over-all reaction (Table II) are not consistent with so simple a model, suggesting that a more complex version of the bond cleavage mechanism may be involved.

It is not known whether the results of the present study are applicable to the case of negatively stained or shadowed particles. It is possible that the negative stain or shadowing material protects the particles to some extent from the damaging effects of the beam. For example, it may be that while ionization and bond cleavage would not be impeded, the escape of low molecular weight decay products would be prevented. This possibility is currently being investigated.

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