ELECTRON IRRADIATION CHANGES IN LIPID LAYER SYSTEMS

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The effect of electron irradiation on lipid layers was studied by electron microscopy, X-ray diffraction analysis and infra-red absorption spectroscopy. By electron microscopy smaller layer spacings are found than by X-ray diffraction. This is shown to be due to the ionizing effect of the electron bombardment, which causes bond rupture, cross linking and loss of material in the molecules of the specimen. Such a disturbance of structure is unavoidable in conventional electron microscopy.

1. INTRODUCTION

Quantitative studies of biological objects with the electron microscope should take into account the effect of electron bombardment on basic substances like proteins, nucleic acids, lipids and carbohydrates. In a previous article (Elbers and Ververgaert, 1965) it was reported that in lipid systems different layer spacings are found depending on whether the investigations are carried out by X-ray diffraction analysis or electron microscopy. The lower value was always found by electron microscopy, which is in accordance with results on similar systems by other authors. Our investigations were done with a homologous series of well defined synthesized phospholipids.

The stability of the specimen was obtained by means of a tricomplex reaction in which the polar groups of fully saturated lecithins are involved (Elbers et al., 1965), and not through the oxidation of unsaturated lipids by osmium tetroxide. It was shown by X-ray analysis that only very slight changes of layer spacing occurred during this preparative treatment. Electron micrographs of thin sections, however, revealed layer spacings which were reduced to up to 20%. Such a difference was ascribed to the heating effect of electron irradiation in the microscope, because it was found by X-ray analysis that heating caused the same reduction in layer spacing.

According to Finean and Millington (1955) heating of phospholipids would induce the formation of new polymorphs based on a tight coiling or a tilt of their hydrocarbon chains with respect to the layer planes, with concomitant reduction

of the spacing. On the other hand, Chapman (1965) favors the view that the hydrocarbon chains take up more of a chaotic configuration and that this causes the long spacings to decrease as the temperature increases. It remains to be proved however that in the electron microscope heat is indeed the cause of the spacing reduction.

There are two types of electron radiation damage in organic matter, namely damage due to temperature rise in the specimen and damage due to ionization (Reimer, 1965). Heat generation is proportional to the intensity of the electron beam in first approximation, besides of being dependent on the geometry of both specimen and beam. Ionization is a dose-dependent effect, in which dose is defined as an electric charge having passed through a unit of object area. It causes bond rupture, cross linking and scission of molecules and carbonization, even if a temperature rise in the object is avoided.

In order to clarify what happens to the lecithins stabilized by tricomplex fixation both possibilities were tested by experiment.

2. MATERIAL AND METHODS

Chromatographically pure samples of three saturated phosphatidylcholines were studied, viz. DL-α-(ditetradecanoyl)-, L-α-(dipentadecanoyl)- and L-α-(dioctadecanoyl) lecithin. Fixation, embedding, electron microscopy and X-ray diffraction were carried out as described by Elbers and Ververgaert (1965). Experiments concerning the temperature effect were done with the Sie-

mens Elmiskop I provided with a pointed fila-
ment cathode and a specimen cooling chamber.
In this way a beam spot size of 1μ diameter
could be used. For a still larger temperature
reduction a specimen cooling device (Elbers,
1966) was applied. Electron micrograph magnifi-
cations were determined according to Elbers and
Pieters (1964).

For the irradiation experiments the Philips
EM 200 was used as an electron generator. To
this end an aluminum target disc of 4 cm^2 was
mounted on an aluminum bar through one of the
portholes, just above the large viewing screen.
The supporting bar was thermally and electri-
cally isolated from the microscope. In this way
the electron current to earth could be measured
directly, while the target was cooled by heat
transfer to liquid nitrogen at the outside of the
microscope. Target temperature was checked
during irradiation by means of a nichrome re-
sistance thermometer fixed between target disc
and supporting bar. Infrared absorption measu-
rements were carried out with a Perkin-Elmer
I.E. Spectrophotometer using a micromethod
with the material made into a KBr disc.

3. EXPERIMENTS AND RESULTS

3.1. *Specimen temperature*

Leisegang (1956) has shown how the specimen
temperature in an electron microscope depends
on the spot size and the current density of the
electron beam (fig. 1). From the diagram it is
seen that with a spot size smaller than 1μ diam-
eter no heating above the specimen stage tem-
perature occurs, even when high beam currents
are used. The same dependency seems to apply
to silicium-dioxide and carbon films of 650 to
400 Å thickness, which are comparable to sec-
tions of the usual thickness. It is for obvious
reasons impossible to measure the temperature
of such a small mass of matter as is irradiated
in the electron microscope.

The lowest beam current which is practicable
at a magnification of $60\,000 \times$ is about 0.1 A/cm^2.
With such a current and a spot size of 1μ diam-
eter sections of the dipentadecanoyllecithin tri-
complex were studied at 80 kV acceleration volt-
age. The micrographs revealed a layer spacing
of 47 Å (S.D. = 4 Å; n = 116). This is the same
as found in the earlier experiments with much
larger spot size (Elbers and Ververgaert, 1965)
and has to be compared with the X-ray repeat
period of 61.5 Å. For comparison the cobaltmo-
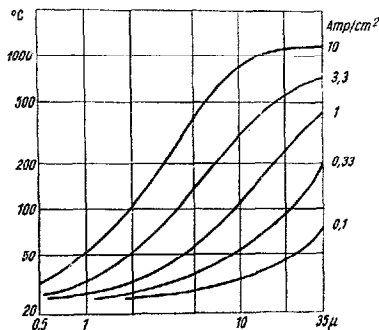lybdate tricomplex of dioctadecanoyllecithin was

Fig. 1. Dependency of temperature on spot size and
beam current in a 100 Å SiO_2 film. Abscissa: radius
of beam spot; ordinate: temperature; at right: beam
current density (after Leisegang, 1956).

studied in the same way. An electron microscope
layer spacing of 51 Å (S.D. = 3.4 Å; n = 42) was
found in this case, in contrast to the X-ray re-
peat period of 69 Å. To make sure that not a
small rise above the specimen stage tempera-
ture could be the cause of such a discrepancy, in
the next experiment the dipentadecanoyllecithin
tricomplex was kept at a temperature of -30°C
by means of the specimen cooling device. The
other irradiation conditions were the same as in
the previous experiments. In this experiment a
layer spacing of 51 Å (S.D. = 2.9 Å; n = 181) was
found, again a considerable difference with the
X-ray period.

3.2. *Electron irradiation effect*

The usual current density on our specimens
at high magnification was first determined in a
Philips E.M. 200 electron microscope. The illu-
mination conditions were chosen so that Ilford
N 60 plates showed sufficient optical density af-
ter 2 sec exposure time at a magnification of
$60\,000 \times$. From the beam spot size and the total
beam current measured at the image screen, the
current density in the specimen was calculated
as $5 \times 10^{-2} \text{ A/cm}^2$. During the photographical
exposure time of 2 sec the specimen therefore
gets an electron dose of 0.1 Asec/cm^2. This is
of course the very minimum dose for electron
microscope structure analysis, because the time
required for actual microscopy, comprising site

location, focusing and through-focus series, is much longer. It was decided to give this minimum dose to an amount of material sufficient for X-ray analysis, in order to find out the effect of irradiation without heating by means of this control method. For practical reasons a 7000 times longer irradiation time, that is about 4 hr, was chosen. The 0.1 Asec/cm^2 dose then is obtained with a total beam current of 30 μA evenly distributed over a target surface of 4 cm^2. The target surface was covered by a suspension of tricomplex floccules made with L-α-(ditetradecanoyl) lecithin. After drying the floccules adhered firmly to the surface. The suspension contained about 1 mg phospholipid. The dried layer thus had a mean thickness of about 2.5 μ, which is readily penetrated by 80 kV electrons.

The heat production of an 80 kV electron beam at 30 μA current is 2.4 W or 0.6 cal/sec, assuming that all electron energy is converted into heat. Aluminum has a heat conductivity of 0.5 cal/sec/^{0}C/cm at 0^{0}C. Under the conditions of irradiation therefore the temperature at the target surface of the 5 mm thick disc rises no more than 0.15^{0}C above the temperature of its lower surface, which was kept at -5 to -10^{0}C. Under such pure electron irradiation conditions, after a dose of 0.1 Asec/cm^2 the white, crystalline fatty lipid material turned into a brown dry powder. The low angle X-ray scattering of this powder now was compared to the diffraction pattern of the original material (fig. 2). This last one shown a clear maximum at 57 Å. In contrast the irradiated material shows a broad principal maximum with a top at 49 Å and ending at 35 Å,

which means a large and generalized reduction of layer spacing. The broad maximum in the X-ray diagram of the irradiated material is entirely in accordance with the large standard deviations which are found in the measurements from electron micrographs.

A comparison of the infra-red spectra of the intact and the irradiated lecithin tricomplex gives us an idea of what happened to various functional groups of the molecules (fig. 3). The intact material gives an I.R. spectrum largely consistent with that of a lecithin of comparable chain length shown by De Haas (1963). The spectrum of the irradiated material differs from this in the following general way: the band due to the carbonyl-group at 1739 cm^{-1} has disappeared, a weak peak at 1700 cm^{-1} indicates double bond formation, the peaks due to CH$_2$ and CH$_3$ groups at 1470 cm^{-1} and 1380 cm^{-1} are much flattened, the two bands due to the phosphate group between 1300 and 1000 cm^{-1} are completely lost as are the bands due to skeletal vibrations between 1000 and 900 cm^{-1} and the small peak due to polymethylene chains at 720 cm^{-1}.

4. DISCUSSION

The experiments provide clear evidence that heat generation in the electron microscope specimen cannot be the cause of the layer spacing reduction. Even with cooling of the specimen this reduction is demonstrated by two independent measuring methods. It must therefore be due to ionization.
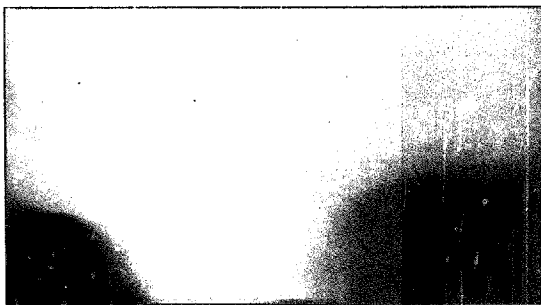

Fig. 2. Low angle X-ray scattering diagram of original (left) and irradiated (right) lecithin tricomplex.
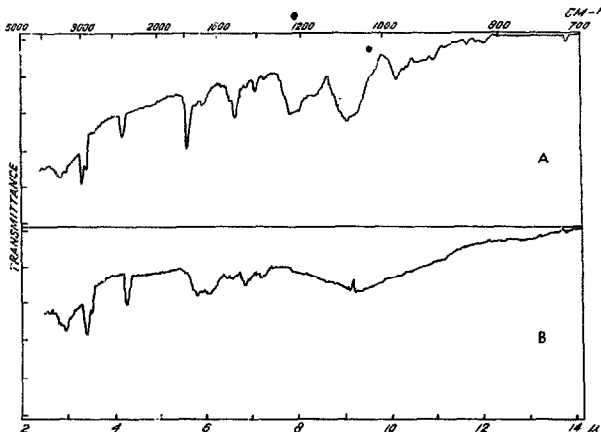
Fig. 3. Infra-red spectra of original (A) and irradiated (B) lecithin tricomplex. Explanation in the text.

Ionization of carbon atoms is the dominant feature in the inelastic collisions of fast electrons and organic molecules. This ionization is followed by secondary chemical reactions, such as cross linking between neighboring molecular chains, scission of chains, double bonding between carbon atoms and extrusion of hydrogen atoms. The rate of the secondary reaction cannot be decreased by cooling (Kobayashi and Sakaoku, 1965). Bahr et al. (1965) followed the effect of these reactions by studying the loss of weight, the infrared spectra and the chemical composition of a number of substances after different electron irradiation doses. For comparison with the phospholipid material the reactions of polyethylene, polyester and polyvinyl formal are of importance.

After a dose of 2×10^{-3} Asec/cm² polyethylene showed a mass loss of 5.3%, consisting of 6% of the original carbon and 20% of the hydrogen content. The I.R. spectrum gives indication of double bond formation and disappearance of the polymethylene bands between 750 and 700 cm⁻¹.

After a dose of 2×10^{-2} Asec/cm² polyester showed a mass loss of 30%, consisting of 15% of the original carbon, 35% of the oxygen and 30% of the hydrogen. From the I.R. spectrum indication is derived of double bond formation, disappearance of carbonyl band and diminishing of skeletal vibrations.

After a dose of 1×10^{-2} Asec/cm² polyvinyl formal turned dark brown, with a mass loss of 50%, consisting of 20% of the original carbon, 80% of the oxygen and 30% of the hydrogen. There was a large contraction effect. The I.R. spectrum reveals the disappearance of bands associated with carbonyl and ester bonds and of skeletal vibration bands.

While the amount of our phospholipid material was insufficient to carry out also determinations of weight loss and chemical composition, the I.R. absorption data clearly indicate that much the same reactions took place in this material. From the X-ray diffraction work (Elbers and Ververgaert, 1965) it was concluded that the lecithin molecules in the tricomplex system are oriented perpendicular to the layer planes, with the end groups fully extended and in line with the hydrocarbon chains. All of the reactions, occurring at electron irradiation, will therefore result in reduction of the layer spacing. This effect is obtained at the very minimum dose for electron micrography, but irradiation damage will start already at much lower doses, dependent on the type of molecules studied (Bahr et al., 1965).

One must expect that the observation of undistorted molecular arrangement in any organic specimen will prove to be impossible with conventional electron microscopy. Caution is therefore warranted in the interpretation of data from

electron micrographs in terms of molecular arrangement.

ACKNOWLEDGEMENTS

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