Electron Microscopy, Electron Probe X-Ray Microanalysis and Microdiffraction of Biopsy Material from Human Liver*

By

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The technique of chemical microanalysis by selected area electron microdiffraction has been already used for elemental analysis of crystal or crystalloid appearing in biological materials. Recently, application of an electron probe x-ray microanalyzer for an elemental analysis has opened new fields of investigation and research in biology. Such application has been capable of rapid, nondestructive quantitative and qualitative analyses of elements upper than about 12 in atomic number, at the light microscopic level, as seen in reviews of Cosslcett ('63) and Tousimis ('66). An application of such analysis to iron compounds in the animal tissue cells has been reported by Lever and Duncumb ('61) in rat duodenum after iron feeding, Boyde et al. ('61) in rodent dental tissue, Yasuzumi et al. ('61) in human erythrocytes, and Yasuzumi in hepatocytes of human biopsy material in a case of pancreatic tumor with obstructive jaundice ('62a), and in nutritive cells of pond snail testis ('62b).

More recently, an advance in instrumental design has made it possible directly to analyze elements of unknown composition appearing in the electron microscope field in a high resolution (Yasuzumi et al., '66). This microanalysis can be carried out in a few minutes, without removing specimen placed in a specimen chamber, but attaching a set of x-ray spectrometer to a specimen chamber.

In the course of an electron microscope study on the liver in about 100 cases of serum hepatitis in humans, ferritin molecules

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have always been observed in several patterns in the cytoplasmic matrix of hepatocytes. It is well known that a ferritin molecule is composed of a protein shell, apoferritin, and a core consisting of inorganic ferric hydroxide in a characteristic pattern (Granick, '51; Farrant, '54; Richter, '57, '59, '63; Daems, '62; Hofmann and Harrison, '63; Haggis, '65; Chescoe and Agar, '66). In the present material, several paracrystalline structures have been occasionally encountered, each of them is not always composed of particulate elements, rather consists of an array of dense lines approximately 60Å wide, arranged parallel to one another, being regularly spaced about 60Å wide. An electron probe x-ray microanalyzer has been applied in order to identify whether elemental components of such a structure consist of an iron compound or other elements. In addition to such analysis, a selected area microdiffraction has been desired to make clear more in detail the character of the observed elemental elements.

Material and Methods

Human liver was obtained by needle biopsy from patients in cases of serum hepatitis, in which several liver function tests were already examined. Blocks ca. 1 mm in thickness were fixed at 4°C for 1 hour in 6.25% glutaraldehyde followed by 1% osmium tetroxide, each being adjusted to pH 7.2 with 0.1 M Na-cacodylate buffer (Sabatin et al., '63). The specimens were then dehydrated in series of increasing concentrations of ethanol and embedded in epoxy Epon resin (Luft, '61). Serial sections were cut on a Porter-Blum microtome or an LKB ultrotome using glass knives. They were mounted on Formvar-coated copper grids. A Hitachi electron microscope, model HU-11 C, was used for electron microscopy, electron probe x-ray microanalysis and selected area microdiffraction. One of a series of serial sections stained either singly with saturated aqueous uranyl acetate (Watson, '58) or doubly with saturated aqueous uranyl followed by lead acetate (Reynolds, '63) was first observed in the electron microscope at a beam potential of 100 kv. The other without staining with any heavy metals was examined by means of an electron probe x-ray microanalyzer, model HXA-1, and then a selected area microdiffraction. A selected area microdiffraction was every time evaluated by taking that of gold crystal. All the specimens were covered with a carbon film, that was especially important for electron probe x-ray microanalysis.
When elemental component of some element was desired to analyze, an x-ray spectrometer was attached to a specimen chamber of the electron microscope in situ. A fine-pointed electron beam about 1μ in diameter was directed at the point with the aid of the electron microscope, being generated with $1 \times 10^{-6}$ amp at 25 kv of an accelerating voltage. The resulting characteristic x-ray emission was collected by a counter of an x-ray spectrometer. The output pulses from the counter modulated the brightness of a beam scanning an oscilloscope screen synchronized with the electron-probe scanning the specimen. By means of the crystal spectrometer, it was possible to select one particular wavelength of x-ray and to collect in the counter the emission of the defined wavelength only. The x-ray spectral analysis was carried out by driving the spectrometer with a synchronous motor and recording the intensity of the emission on a pen recorder.

Observations

Most of the cytoplasm of hepatocytes contain large aggregates of electron opaque particles, approximately 60A in diameter, some of the aggregates measuring up to 1.4μ in cross-section. These particles are occasionally found in electron-lucent vesicular bodies. Several dense bodies of varying size and shape appear seldom mixed with the dense 60-A particles. A representative section of such structure is pictured in Fig. 1. The cytoplasm of these hepatocytes contains numerous dense glycogen granules, a few granular endoplasmic reticulum, denser bodies in different sizes and shapes, lysosomes, free ribosomes and mitochondria provided with a few cristae and homogeneously fine granular matrix of intermediate density. Although a few dense granules are often observed in the mitochondrial matrix, which have been first described by Sjöstrand and Rhodin ('53), no dense 60-A particles are found in the matrix.

Two types of the glycogen granules are clearly distinguishable morphologically: single granules (β particles) approximately 200A in diameter and rosettes (α particles) comprised of varying numbers of about 200-A particles, with an aggregate diameter of 500 to 1000A. Mixtures of these particles occur in the hepatocyte cytoplasm, though α particles tend to predominate in cells containing a small number of aggregate of dense 60-A particles.

A similar part in another one of serial sections as an area
containing an aggregate of dense 60-A particles, without staining with heavy metals, has been analyzed by an electron probe x-ray microanalyzer. An Fe-Kα spectrum has been obtained, showing that such dense particle aggregate contains a large amount of iron elements (Fig. 2).

The dense 60-A particles appear sometimes diffusely dispersed in the cytoplasmic matrix in which glycogen granules are no more visible (Fig. 3). The hepatocytes devoid completely of glycogen granules contain often numerous dense 60-A particles aggregated in the cytoplasmic matrix, without being surrounded by a limiting membrane. These are considered to be disordered crystalline arrays of ferritin (Fig. 4). As the aggregation proceeds, it becomes to show a peculiar paracrystalline structure in arrangement of particles (Fig. 5). The aggregated particles appear to be too dense to observe their fine structure, but isolated ones reveal a characteristic fine ferritin pattern, being composed of sub-units measuring 25A in diameter at higher magnification (Fig. 6).

A similar part in another one of serial sections as seen in Fig. 4 has been analyzed by the aid of an electron probe x-ray microanalyzer and a selected area microdiffraction. An Fe-Kα spectrum has been obtained from a large aggregate of electron opaque particles (Fig. 7). The same part has been analyzed by a selected area microdiffraction and the interplanar distances observed are calculated on the basis of diffraction pattern of standard gold crystal (Fig. 8): 2.52, 2.27, 1.98, 1.75 and 1.46 dA.

A small cluster of irregularly shaped, ill-defined dense bodies are observed in some part of the cytoplasm of a hepatocyte (Fig. 9). In higher magnification, a peculiar structure has been observed in these bodies, in which can be seen dense, interrupted lines arranged parallel to one another: center to center spacings are measured to be 100 to 110A (Fig. 10).

An electron probe x-ray microanalysis has shown that the elements constituting the dense lines consist of iron compounds (Fig. 11). Interplanar distances of selected area microdiffraction of these iron compounds (Fig. 12) are calculated to be at 2.51, 2.23, 2.00, 1.70, 1.50 and 1.40 dA.

The interplanar distances of diffraction patterns demonstrated in Figs. 8 and 12 are compared with those of β-iron oxide hydrate reported in “X-Ray Powder Data File” (H a n a w a l t, ’38; M c K a y, ’60) (Table 1).

The results given in Table 1 indicate that the interplanar
Table 1. Interplanar distances (dA) of diffraction patterns of ferritin molecules and \( \beta \)-ferric oxide mono-hydrate.

<table>
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<tr>
<th>Ferritin molecules observed (Fig. 8)</th>
<th>( \beta )-iron oxide mono-hydrate (H a n a w a l t)</th>
<th>Ferritin molecules observed (Fig. 12)</th>
<th>( \beta )-iron oxide mono-hydrate (M c K a y)</th>
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Distances of the observed ferritin molecules are similar to those of \( \beta \)-iron oxide mono-hydrate reported in "X-Ray Powder Data File". A slight disparity of the distances mentioned above may be dependent on difference of dehydration or of other preparation techniques of specimens, as described by H a r r i s o n et al. (67).

**Discussion**

Ferritin molecules in several distributions or aggregations have been observed in the cytoplasm of hepatocytes in cases of serum hepatitis. In the area where glycogen granules are found, the compactly aggregated forms of ferritin molecules are present in the electron-lucent vesicular bodies. Such bodies may be derivatives of lysosomes or mitochondria. The glycogen granules are no more visible when ferritin molecules appear either dispersed through the cytoplasmic matrix or aggregated in a paracrystalline array in the cytoplasm. Therefore, the occurrence of ferritin molecules may induce the disappearance of glycogen granules.

It is of interest that the fine structure of ferritin molecules present remarkable analogies to that of virus particles, since the latter have in general a protein shell and a distinct core containing nucleic acids. It is well known that virus particles appear occasionally aggregated in a paracrystalline array, for example, as seen in those found in the cytoplasm of human embryonic lung cells grown in tissue culture, in which the virus was isolated from the feces of a patient during an epidemic of infectious hepatitis (S t e i n e r et al., '65). Such appearance seems to be similar to a parallel arrangement of dense lines spaced in a regular way (Fig. 10), though their size is different from that of the virus particles (S t e i n e r et al., '65).
The microchemical analysis of components constituting such a particular structure as seen in Fig. 10 has been done by means of an electron probe x-ray microanalyzer. The results have shown a spectrum formed by the Fe-Ke emission. An improvement of equipment has made it possible to analyze elements of unknown composition resolvable in the electron microscope directly with an electron probe x-ray microanalyzer. Therefore, such microchemical analysis has proved to be useful in biology.

Through several patterns of distributions and aggregations of ferritin molecules appearing in a hepatocyte, their development is believed as follows. Ferritin molecules appear diffusely dispersed in the cytoplasmic matrix or aggregated in a certain cytoplasmic area or in cell organelles (Richter, '57; Daems, '62; Yasuzumi, '62a). In early stages of their aggregation, they seem to present at random. As their aggregation develops, they are situated to be in a regular arrangement, that may be a result of dehydration. Moreover, as the development of aggregate proceeds, the particles are connected to show a linear feature, keeping their original size, though the mechanism of such formation remains unknown. The present microdiffraction patterns have shown that the ferritin molecules appearing in the hepatocyte cytoplasm may be composed of p-ferric oxide monohydrate.

Ferritin is widely distributed through the animal kingdom, not only vertebrates, but also certain intertebrates, suggesting that ferritin is synthesized by several cells in various animal species (Favard and Carasso, '58; Muir, '60; Roche et al., '61; Yasuzumi, '62b). This fact seems to indicate that the occurrence of ferritin in hepatocytes has an important physiological significance as to their metabolism, as reported by several authors (Mazur et al., '58; Mazur et al., '60; Mazur and Carlton, '65). However, so far as the present study is concerned, it is not clear whether there is a causal or necessary relationship between the occurrence of ferritin molecules in a large amount in hepatocytes and viral infection in serum hepatitis.

Summary

Ultrastructural and microchemical analyses were made on dense particles, approximately 60 A in diameter, appearing in the cytoplasm of hepatocytes of human biopsy material in all the cases of serum
hepatitis by means of electron microscopy, electron probe x-ray microanalysis and selected area microdiffraction. The particles were found in the electron-lucent vesicular bodies, or widely scattered in the cytoplasmic matrix, or aggregated in several forms and even in a paracrystalline feature. The isolated particles were clearly identified to be ferritin molecules on the basis of their size and characteristic sub-unit structure, while the aggregated ones were not always revealed to be ferritin molecules by electron microscopy. Electron probe x-ray microanalysis and selected area microdiffraction made it possible to analyze that the dense particles aggregated in a paracrystalline feature, or in a linear pattern arranged parallel to each other, are of iron compounds consisting of β-ferric oxide monohydrate.

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References


Explanation of Figures

Fig. 1 shows an aggregate of dense particles approximately 60A in diameter in an electron-lucent vesicular body. Remarkably dense granules (DG) appear in the body, and dense bodies (DB) in the cytoplasm at the right side as well as at the right lower corner of the field. Numerous glycogen granules appear in the cytoplasmic matrix, many of them showing a rosette form. Free ribosomes (R) are clearly identified in a more clear rosette form than the glycogen granules. Mitochondria (M) contain a few dense granules in their matrix. Lysosomes (LY) can be seen at the upper side of the figure. The endoplasmic reticulum appears in a small amount. The boundary of adjacent cells is not clear, but the desmosome (DS) is clearly visible. x 58,000.

Fig. 2. Fe-Kα spectrum obtained from a similar aggregate of dense particles as shown in Fig. 1.

Fig. 3 demonstrates numerous elongated or irregularly swollen microvilli protruding into the Disse space. The cytoplasmic matrix and microvilli are diffusely filled with dense particles about 60A in diameter. But, they tend to aggregate in certain areas (arrows). Roughly oval-shaped profiles of dense bodies (DB) are visible in the cytoplasm. Mitochondria (M) degenerating in their size and structure appear at the right lower corner. x 64,000.

Fig. 4. An aggregate of dense particles approximately 60A in diameter in the cytoplasm, showing a tendency to linear arrangement of the particles. x 200,000.

Fig. 5. Part of an irregularly outlined aggregate of electron dense 60-A particles, without being surrounded by a limiting membrane. The particles are arranged in a paracrystalline form at several points. x 180,000.

Fig. 6. A higher magnification of two particles marked by the arrow in Fig. 5. x 380,000.

Fig. 7. Fe-Kα spectrum recorded from a similar part as shown in Fig. 4.

Fig. 8. A selected area microdiffraction pattern obtained from the same part as observed in Fig. 7.

Fig. 9. A cluster of irregularly shaped, ill-defined bodies appearing in the cytoplasm of a hepatocyte. x 28,000.

Fig. 10. A higher magnification of the part marked by the arrow in Fig. 9. The bodies are not homogeneously dense, but they are composed of a parallel arrangement of dense lines which seem to have been originated from array of dense particles approximately 60A in diameter. x 344,000.

Fig. 11. Fe-Kα spectrum at a similar part as demonstrated in Fig. 9.

Fig. 12. A selected area microdiffraction pattern obtained from the same part as observed in Fig. 11.
Plate I

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Plate II

Fe-Kα³ (5.81 Å)

Counting per Second

Diffusion Angle

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Plate IX

Diffusion Angle

Fe-Kα³(5.81 Å)

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Plate X

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