Electron Microscopy: Cytology of Cell Fractions

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Since the inception of the technique of differential centrifugation, biochemists have been concerned with the cytologic characterization of the sedimented fractions. In homogenates of liver, the organ generally chosen for such studies, they were able to identify unbroken cells, erythrocytes, nuclei, and mitochondria with the usual light microscope. The phase-contrast microscope made analyses of these structures considerably more reliable, and it made possible the detection of microsomes (*1*) and the identification of structures such as bile canaliculi (*2*). Electron microscopy of thin sections now brings within reach virtually complete cytologic characterization of all particulate fractions.

It is the purpose of this article to indicate how in a short time, beginning with the studies of Palade (*3*) and Palade and Siekevitz (*4*), the electron microscope has contributed significantly to the study of cell fractions. Our work (*5–8*), like that of others which is to be discussed, has dealt thus far with normal rat liver. Figure 1 is a diagram of the parenchymatous cell of this organ, in which are shown the structures revealed by electron-microscope studies to which I shall refer.

Nuclear Fraction

Of all the fractions obtained from the usual sucrose homogenates, the nuclear fraction is the most contaminated. In addition to nuclei, variable numbers of unbroken cells, erythrocytes, cell membranes (including bile canaliculi), and mitochondria can readily be seen with the phase-contrast microscope. Phase-contrast microscopy also gives some indication of extraction of materials from the nuclei, particularly when the nuclear fraction is washed. Surrounding each free nucleus is an irregular clear zone into which cytoplasmic particles do not move, suggesting the presence of a gel-like material.

Thus far we have obtained the best electron micrographs of this fraction when it was isolated from 7.3-percent polyvinylpyrollidone-0.25*M* sucrose homogenates (*8*). Following fixation in 7.3-percent polyvinylpyrollidone, 0.25*M* sucrose, and 2-percent osmium tetroxide at *p*H 7.0 (*6*), the nuclei appear like those of tissue sections. The nucleoli show prominently (*9–11*). The nuclear membrane, still clearly double, shows regularly spaced gaps distributed like the nuclear "pores" described in tissue sections (*11–14;* but see *15, 16*). The manipulative procedures appear to have enlarged the "pores," and in many of them a finely granular material is seen which is also present within and just outside the nucleus. This probably corresponds to the gel-like material seen around the nuclei with phase-contrast microscopy; it may contain the protein which Pollister and Leuchtenberger (*17*) showed to be lost from nuclei during their isolation from aqueous media.

Among the non-nuclear components of the fraction, one can readily discern in the thin sections the mitochondria and microsomes with their typical fine structure described in subsequent paragraphs. Also present are many small membranous structures that require further study. Some seem to be the microvilli first revealed by electron microscopy inside the bile canaliculi in tissue sections (*10, 11, 18*).

Mitochondria

Mitochondria were isolated from liver for the first time by Bensley and Hoerr in 1934 (*19*). Shortly thereafter, Claude (*20–22*) reported the separation of submicroscopic granules as well as "large granules." It was Claude's work (*20–23*) that made differential centrifugation one of the most popular techniques of modern biochemistry. Yet, for reasons discussed by Hoerr (*24*), it left the cytologic nature of the "large granule" fraction unclarified. In 1948 Hogeboom, Schneider, and Palade (*25*) found that the granules of the fraction were stained by Janus green B. They were also able to show that, when they were isolated from hypertonic sucrose homogenates, these granules were often rod-shaped, as they are in intact cells. Since that time, the fraction has been referred to as "mitochondrial." The notable success of biochemists in demonstrating that the mitochondrial fraction carried on oxidative phosphorylation and possessed other important enzymic properties relegated to the background the problems of alteration of the granules during isolation and the contamination of the fraction by other cytoplasmic constituents.

The different shapes and sizes of mitochondria isolated from different media could be determined by light microscopy. With phase-contrast microscopy, the alteration of mitochondria could be followed—for example, when the hypertonic sucrose rods swelled into large spheres containing crescent-shaped areas at one pole. With the electron microscope it is possible to judge the loss of material from their interiors and the changes in outer and inner membranes (*26–28*). Witter, Watson, and Cottone (*29*) were the first to publish electron micrographs of the alteration of fine structure of mitochondria isolated from isotonic and hypertonic sucrose (*30*); from their studies, these authors suggested 0.44*M* sucrose that has been brought to *p*H 6.2 with citrate as a medium from which to isolate the mitochondria. On the basis of similar studies, I have suggested (*5, 6, 8*) 7.3-percent polyvinylpyrollidone plus 0.25*M* sucrose at *p*H 7.6 to 7.8.

Electron microscopy also makes possible unequivocal decisions concerning the contamination of mitochondrial fractions. For example, the difference in opinion between us (*1*) and Laird *et al.* (*31*) was readily resolved by studying thin sections of purified "fluffy layer" (*5, 6*).

By evaluating the purity of the mitochondrial fractions and the state of mitochondrial preservation, electron microscopy can go far toward resolving the question of whether in liver the mitochondria are biochemically heterogeneous. I have recently (*6*) reviewed the literature and have expressed the opinion that the biochemical data are as yet inadequate to establish such heterogeneity.

I have noted (*6*) that rat-liver homogenates contain many small mitochondria, the average dimensions of which, in the sections, are 270 and 610 millimicrons. These appear to have a greater number of electron-dense granules, particularly in relation to size, than the other mitochondria. If, as Weiss (*32*) suggests, these granules bind potassium in an osmotically inactive form, their greater number may be related to an apparently greater resistance of these mitochondria to osmotic swelling during homogenization and washing in hypertonic sucrose (*6*). Chemical analyses of these small mito-

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chondria would be highly desirable, but we have been unable as yet to prepare them free of microsomes.

It is interesting to consider whether these small mitochondria of liver correspond to the "ultrachondrioma" described in leucocytes and other cells by Oberling and coworkers (*33, 34*). On the basis of their shape, osmophilia, and polymorphism, and on the basis of the existence of transitional forms, these authors consider the "ultrachondrioma" elements to be small mitochondria. It is to be expected that thin sections will be studied to determine whether their fine structure is that of mitochondria. The width of most "ultrachondrioma" elements is about 150 millimicrons or less. The mean width of the small liver mitochondria is considerably greater (270 millimicrons), but about 20 percent are only 160 to 180 millimicrons wide. In evaluating the difference between the two types of particle, we may have to consider not only the different cells from which they are derived but also the fact that the cells showing "ultrachondrioma" grew flattened on Formvar films and were studied as whole mounts.

Microsomes

Only with electron microscopy of thin sections is it possible to relate the microsomes to cytologic entities with any degree of confidence. Although much remains to be done before we can describe the fine structure of the entire microsome fraction, even of normal liver, already it is possible, *for this tissue,* to attach cytologic meaning to a term which, from the time Claude (*21*) adopted it for the "small granules," has remained simply an operational name for the granules that remain in suspension after the "large granules" have been sedimented. In adopting the noncommital term for the fraction, Claude (*22*) pointed out that "a more descriptive term may be indicated when the nature and function of the submicroscopic substance is better understood."

Studies of the microsome fraction of rat liver by Palade and Siekevitz (*4*) and later ones by myself and others (*35*) show the major constituent of the fraction to be the membranes on which are found the small dense particles seen by Weiss (*36*) and described in fine detail by Palade (*37*). When the microsomes are isolated in isotonic sucrose and fixed in isotonic sucrose and osmium tetroxide, the membranes appear as tiny granule-studded sacs. However, when they are isolated in hypertonic sucrose or in polyvinylpyrollidone-sucrose and fixed in the same media plus osmium tetroxide, the membranes are flattened and virtually identical, except for length, with the basophilic material that is seen in liver sections (*6, 8, 10, 11, 27, 38*).

Electron microscopists have divided sharply on the question of whether to speak of this basophilic material as "ergastoplasm" (*36, 38, 39*) or "endoplasmic reticulum" (*37, 40–42*). Those who preferred the latter name pointed to

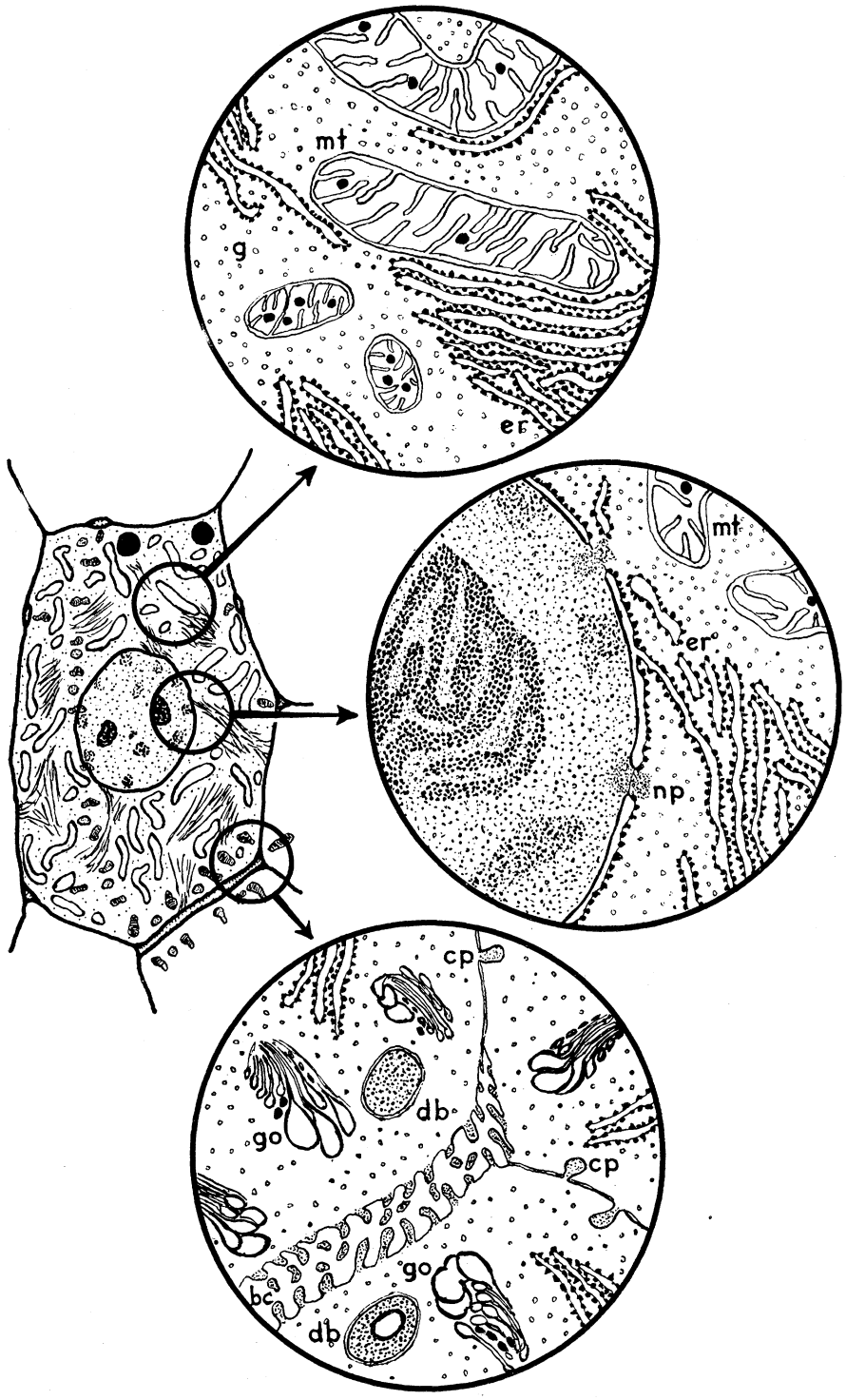

Fig. 1. Schematic representation of a parenchymatous cell of rat liver. The fine structure depicted is based largely on the following investigations: mitochondria (*mt*) (*26–28*); basophilic material (*er*) (*10, 11, 27, 38, 41*); glycogen (*g*) (*11*); nucleolus (*9–11*); nuclear membrane (*12*); nuclear "pores" (*np*) (*11–16*); Golgi apparatus (*go*) (*11, 18, 39, 48, 49, 51–53*); "dense bodies" (*db*) (*6, 7, 18*); interlocking cell processes (*cp*) (*11*); and bile canaliculus (*bc*) (*10, 11, 18*). Bile canaliculi are shown sectioned longitudinally at the right bottom of cell and transversely in five other regions (*2*). The linear arrangement of Golgi material to the left of the nucleus suggests a canaliculus oriented along the length of the cell but lying outside the plane of section. Lipid droplets are indicated by two solid spheres at one pole of the cell.

the specialized, often misleading, meanings that were given to the term *ergastoplasm* early in this century. This could, however, be countered with the observations (i) that, although Garnier (*43*) used the term for the filamentous structures of gland cells and oocytes, it was subsequently employed by French cytologists more generally for the basophilic substance of cells, and (ii) that many biological terms have undergone changes in meaning without loss of usefulness. On the other hand, those who preferred *ergastoplasm* to *endoplasmic reticulum* pointed to the uncertainty that the membranes, except in unusual cases, constitute a reticulum. However, apparently separate elements may be interconnected outside the plane of section (*42*) and, if Palade's description (*42*) of a continuity between this membranous system and the outer cell membrane proves to be of general occurrence, the term *reticulum,* if not the term *endoplasmic,* would emphasize a relationship of great physiological importance.

Agreement on terminology, sometimes made more difficult by considerations of national pride and human personality, appears to be close at hand, as we rapidly learn more about the membrane systems of cells. Porter's work (*44*) suggests that all cells possess a similar basic component—a vacuolar system of great complexity—and that in different cells this system shows varying degrees of continuity and specialization. Thus, according to this view, "ergastoplasm" is essentially a specialized type of "endoplasmic reticulum" characterized by the presence of basophilic granules on its surface.

I am suggesting that the term generally accepted by electron microscopists for the basophilic material of rat liver, whether it is *ergastoplasm* or another term, also be used for the microsome fraction of normal rat liver. What Harel and Oberling (*34*) have emphasized concerning "optical and electronical" cytologies applies also to biochemical cytology—they are all one cytology. Our knowledge of the "small" particles has advanced essentially to the same position as did that of the larger ones in 1948, when Hogeboom, Schneider, and Palade (*25*) demonstrated that many of the "large granules" had the shape and Janus-green stainability of mitochondria (*45*).

Undivided microsomal fractions of rat liver do not consist solely of granule-studded membranes. They contain considerable numbers of small nongranular membranes which may perhaps be derived from structures as diverse as Golgi apparatus, microvilli of bile canaliculi, and mitochondrial membranes. However, it is easier to obtain relatively purified granular membrane fractions by centrifugation procedures than mitochondrial fractions of equal purity. Not only are mitochondrial fractions contaminated by microsomes, but they generally contain particles such as "lysosomes." It is clearly desirable to separate the nongranular from the granular membranes of the microsome fraction (*46*), just as it is desirable to separate lysosomes and other particles from the mitochondrial fraction. Future work will indicate whether in other tissues the granule-studded membranes also constitute the major component of the fraction or whether other cytologic structures are relatively more important.

Golgi Apparatus

The only description of isolated Golgi material is that of Schneider, Dalton, and coworkers (*47*), with epididymis as the source.

No cytoplasmic structure has been debated with more passion among cytologists than the Golgi apparatus, nor has any structure been more frequently declared nonexistent. Thanks to the steadfast work of Dalton (*39, 48, 49*) and the more recent contributions of Sjöstrand and Hanzon (*50*), Haguenau and Bernhard (*51*), and others (*11, 18, 52, 53*), there can no longer be any doubt that a definite morphologic entity exists in the Golgi zone, at least of vertebrate cells. This consists of a system of smooth membranes, small granules and larger vacuoles. Baker (*54*) contends that the material generally called Golgi apparatus in invertebrates (and in the sympathetic neurone of the rabbit) is not homologous to the structure described by electron microscopists; electron micrographs of these cells could quickly settle the issue.

Electron microscopy also gives some support for the earlier speculations of cytologists that the Golgi material played some role in secretion and intracellular transport (*39, 50, 51, 53*). This makes further work on the isolation of this material for chemical analysis highly desirable. Such work with liver will be difficult, for the Golgi material is not abundant in this tissue. It has the same fine structure as in other tissues (*11, 18, 51*). Although it lies near the bile canaliculi, light microscope studies (*2*) indicate no connections between Golgi material and bile canaliculi such as are described by Gatenby (*55*). Electron microscopy will help determine what happens to the Golgi apparatus during homogenization, whether any remains with the bile canaliculi found in the nuclear fraction (*2*), and when isolated fractions are sufficiently pure to make chemical data meaningful. Polyvinylpyrollidone plus sucrose may be a helpful medium for maintaining the integrity of the Golgi material during isolation (*8*).

Other Cytoplasmic Granules

The recent work of De Duve and coworkers (*56, 57*) points to the possibility that some intracellular enzymes may be localized in cytoplasmic particles other than mitochondria and microsomes. They have found that a fraction which contains no more than 4 percent of the cell's nitrogen contains the major part of the activity of acid phosphatase and four other hydrolytic enzymes. To account for the biochemical data, they postulated the existence in this fraction of dense particles between 0.13 and 0.8 micron in diameter and surrounded by a semipermeable membrane, for which they suggested the name *lysosome.* Electron micrographs of these fractions have revealed the presence of hitherto undescribed particles ("dense bodies") that fit these predictions (*6, 7*). They have a mean length of 0.37 micron, contain tiny electron-dense granules, and some at least, possess internal cavities and external membranes. However, in the absence of highly purified fractions, it can not be asserted that these particles do in fact correspond to the postulated "lysosomes." These particles have also been seen near the bile canaliculi in electron micrographs of liver sections prepared in Bernhard's laboratory (*7, 18*). It is possible that the same or similar granules account for the distribution shown along the bile canaliculi of acid phosphatase and esterase, another hydrolytic enzyme, in Holt's cytochemical preparations (*58*).

Summary

It should be evident from this brief account that electron microscopy of thin sections is an invaluable asset in the study of fractions isolated by differential centrifugation. I have tried to indicate how the integrity of particles, purity of fractions, and the existence of new particles can be established through its use.

I have also suggested the desirability of a common terminology for all cytology—classic, electron-microscopic, and biochemical. Some have expressed the opinion that neutral terms such as *alpha, beta,* and *gamma membranes* (*59*) are more useful than *ergastoplasm, Golgi apparatus,* and so forth. As helpful as such neutral terms may be in *describing* intracellular structures, they do not appear to me to substitute for historically rooted cytological names.

Note added in proof: Since this article went to press there has appeared an important article by G. E. Palade and P. Siekevitz (*60*). These authors consider that the vesicles without granules found in the microsome fraction were "probably derived from the smooth surfaced

parts of the endoplasmic reticulum." The latter were found to be continuous with the granule-studded membranes; "the two varieties of profiles represent local differentiation within a common system." The authors confirm the finding of Rouiller (*18*) and Novikoff *et al.* (*7*) of the dense bodies adjacent to the bile canaliculi and describe their presence in the microsome fraction as a "minor component."

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46. This is especially important in order to resolve the apparent differences between Palade and Siekevitz (*4*) and Littlefield *et al.* [*J. Biol. Chem.* 217, 111 (1955)] on the one hand, and Hogeboom and Kuff [*Federation Proc.* 14, 633 (1955)] and Dalton (*39*) on the other. The former authors have found ribonucleic acid to be localized in the small granular component of the basophilic material, and the latter authors consider ribonucleic acid to be present also in some granule-free membranes, possibly those of the Golgi apparatus.
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R. R. Bensley, Cytologist

On 11 June Robert Russell Bensley died, aged 88 years. His was a full and enjoyable life. He was born on a farm near Hamilton, Ontario, on 13 November 1867 of an English-Canadian father and an Irish mother. Dr. Bensley liked to think of himself as Irish and, in truth, his cheerfulness—one might almost say buoyancy—generosity, and insight in character reading were remarkable. Probably his early family environment, supplemented by his training in English and the classics at school and the University of Toronto, contributed to his mastery of the English language and the clearness of his speeches and his writings.

In his third year of college he suffered a severe gunshot wound of the leg. He saved his life by promptly applying a tourniquet. Amputation was performed on the dining room table of the farm. Gangrene set in. There was another amputation high up in the thigh. Thrombophlebitis, septicemia, and bacterial endocarditis developed. There was much pain. The physician left the young patient to die with a pound of opium. But Bensley overcame the craving induced. During a long year of convalescence he read widely and studied all the farm animals and plants, with the help of a microscope bought for him by his father.

The year 1892 was another eventful one for Bensley. He graduated from Toronto in medicine, married Cariella May, and began the practice of medicine but continued to teach in the department of biology. His home for the rest of his life was a happy one, presided over by a devoted wife and blessed with children and eventually grandchildren.

Beginning in 1901 Bensley served in the department of anatomy of the University of Chicago, first as assistant professor and later as head, until his retirement in 1933. His life was bounded