SUBCELLULAR PARTICLES

A SYMPOSIUM HELD DURING THE MEETING OF THE SOCIETY OF GENERAL PHYSIOLOGISTS AT THE MARINE BIOLOGICAL LABORATORY, WOODS HOLE, MASSACHUSETTS, JUNE 9–11, 1958

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THE RONALD PRESS COMPANY • NEW YORK

LIBRARY OF CONGRESS
CATALOGUE CARD NUMBER
59–11056

PRINTED IN THE UNITED STATES OF AMERICA BY
THE LORD BALTIMORE PRESS, INC., BALTIMORE, MD.

Preface

THE SUBJECT of this year's Symposium of the Society of General Physiologists, 'Subcellular Particles,' was selected by the Council of the Society. In the organization of this symposium, the aim was two-fold: first, to bring to the attention of the variegated membership of the Society the most recent work of the foremost investigators studying subcellular particles; and second, to emphasize the structural aspects of subcellular particles as related to their function, especially with regard to the properties of the heterogeneous system created by the very presence of the particulate material within the cell. It would be presumptuous to say that these aims were achieved with any degree of success, especially in the case of the second part of the over-all aim, where a large area of ignorance faces the experimenter.

The post-war years will, I believe, be considered a 'Golden Era' in the biological sciences and certainly in the study of cell inclusions. Tremendous advances have been made in elucidating the various activities of subcellular structures, due primarily to advances in technology and the inspiration of new concepts and new information from allied fields, as microbiology. Thus improvements in, and the coordination of, techniques in ultracentrifugation, electron microscopy and microchemical analysis have made possible a more definitive correlation between the particles or the parts thereof and their activities within the cell. Likewise, new information about important biochemical substances, as the nucleic acids, have influenced the trend of thought as to the function of intracellular structures. Yet, essentially, this type of progress of knowledge in this field does not shed light on such questions as 1) why are structural units necessary at all, and 2) what effect does structure have on chemical or biochemical conditions and/or reactions within the cell?

However, certain consistent generalities can be observed as the result of the sharp attention paid to intracellular structures. For example, structures seem to be involved in those situations where the cell requires protection from disrupting agents which, nonetheless, the cell must include as a part of its over-all organization in order to maintain a specialized function. Thus enzymes which, if allowed loose in the cell, would cause autolysis would be included in this category. Several papers in this volume point to this aspect of cellular structure and function.

Structure also seems to be necessary in the general situation where synthesis of substances is taking place. Here, however, in spite of the speculations concerning 'templates' the situation is not so simple nor so clear that the conclusion can be stated with any finality. A third and what seems to be a most important requirement for structure within the cell is found in the case of cellular activities resulting

in the transformation of energy. Thus the chloroplast structure plays a prominent part in the transformation of radiant energy into the potential energy of oxidation-reduction reactions or the chemical energy of 'high energy' phosphate bonds (photosynthetic phosphorylation).

It is to be expected that reactions so intrinsically a part of the cell's being would have their characteristics determined by the fact that the components of the reactions appear to be part of a highly-organized and definite structure, and not freely in solution subject to classical kinetics. Here again surprisingly little information is available. Yet an awareness of the effect of a heterogeneous milieu is of paramount importance if we wish to appreciate in full the activities and potentialities of the cell.

It is gratifying to note that while these problems are as yet far from being understood, many of the papers in this symposium are directly concerned with them, and through ingenious experiments a great deal of new knowledge has been brought forth bearing on these questions. To this extent it is hoped that this volume will be of benefit to all those who consider themselves students of the cell.

The Society of General Physiologists gratefully acknowledges the support in the form of a grant from the National Institutes of Health. I should like to express my appreciation to Dr. William D. McElroy, Dr. Eric G. Ball, and Dr. Van R. Potter for having served as chairmen in the three sessions of the symposium, which they did with skill and discretion. My appreciation is also extended to the administration and staff of the Marine Biological Laboratory for their unfailing cooperation and efforts to make the meeting a success. Finally, my thanks go to Mr. Robert Kirchen and Miss Lillian Blaschke for serving as volunteer messengers during the symposium, and to Mrs. Sally Hayashi and the staff of the American Physiological Society, who deserve the major credit for the editing and assembling of this volume.

TERU HAYASHI
February, 1959

Contents

Approaches to the In Vivo Function of Subcellular Particles[1]

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THANKS TO BRACHET (3) we have available a popular term which includes the subject matter of this conference, *Biochemical Cytology*—or, if biochemists prefer, *Cytologic Biochemistry*.

Perhaps it is a sign of maturity of the field that one notes a growing tolerance by specialists in one area of the vast domain of biochemical cytology for the pursuits of those in other areas. There are fewer 'gloomy critics,' as I like to call those people with essentially destructive comments on a technique which they do not themselves employ.

We can all recall the categorical assertion that hope for significant information concerning the *in vivo* function of subcellular particles was lost the moment the cell was disrupted or homogenized. Or, that the use of aqueous media like sucrose could yield only misleading particles, especially worthless nuclei. Or, that oxidative phosphorylation could never be retained once the organized structure of the complete mitochondrion was broken. Or, that electron microscopy was one huge blunder, based as it was on osmium artifacts. Or, that quantitative microspectrophotometry of stained tissue sections was deprived of meaning by the marked structural heterogeneity of the subcellular particles. Or, that enzyme destruction by fixative, and diffusion of reaction product during incubation, made staining methods worthless for demonstrating the intracellular *in situ* localization of enzymes, particularly important ones.

It might be asserted by some that the 'gloomy critics' have helped focus on the pitfalls of biochemical cytology techniques. Others may consider this debatable and contend that it is data rather than critics which move investigators to refine their methods. As the field of biochemical cytology has developed, not only have technical refinements been introduced, but increasingly within the same, or nearby, laboratories integrated studies are pursued, with two or more techniques applied to the same problem.

[1] Work from our laboratory has been supported by grants from the American Cancer Society, Inc.; Public Health Service, U.S. Dept. of Health, Education and Welfare; and the National Science Foundation.

PHASE CONTRAST MICROSCOPY APPLIED TO LIVING AND SURVIVING CELLS

Our survey of techniques of biochemical cytology naturally begins with the living cell. The tissue culture cells virtually speak for themselves in the excellent motion pictures taken with the phase-contrast microscope or interference microscope. Among the films we have seen are those of Dr. Gey, showing remarkable mitochondrial movement, cell membrane activity and pinocytosis (14); Dr. Biesele, showing the speeding of mitochondrial movement by coenzyme A addition and the accentuation of the tendency of mitochondria in the mouse fibroblasts to join end to end (55);[2] Drs. Frederic and Chèvremont, showing the dramatic changes in mitochondria induced by a variety of drugs (6); Dr. Rose, showing the remarkable transformations of pinocytosis vacuoles in a variant of the HeLa cell (45);[2] and Drs. Bloom and Zirkle, showing mitosis in amphibian fibroblasts, with chromosomes and kinetochore evident as well as the extensive surface bubbling at late anaphase and telophase.[2]

Even tissue culture has a limitation we encounter with other techniques: it reveals the *capacities* of the cultured cells, but perhaps not the *actualities* of these cells in the organized structure of the multicellular organism. The specialized milieu in which they are grown is quite different from that encountered naturally by cells embedded in tissue mucopolysaccharide or wedged in tightly among neighboring cells, as in epithelium—cells always under the controlling neural, hormonal and neurohumoral influences of the organism. Thus, the chromosomal changes which many cells in culture undergo so readily may occur very rarely within the tissue. The very extensive pinocytosis or the surface bubbling at late mitotic anaphase and telophase may be exaggerations of surface changes which cells display in the organism.

This in no way minimizes the importance of such films in emphasizing the dynamic nature of the cell. They are balancing forces needed when we reflect upon the static electron micrographs of mitochondria or of plasma membranes in cells, or when we consider the biochemist's descriptions of the multi-enzyme machinery of mitochondria, such as Dr. Green's brilliant description in this volume (17) of the shuttling of small molecules within the lipoprotein matrix. The description of the cell's constant dynamism in biochemical terms is the great challenge for future biochemical cytologists.

Phase-contrast micrography can be of great value to biochemists and others without cinemaphotography equipment and even without truly living cells. I have in mind examination of fractions isolated by differential centrifugation, or of surviving (or dying) cells, as in homogenates.

In figures 1 and 2 are phase contrast photographs of cells in homogenates. It is evident that in liver cells the mitochondria are large and numerous, while in cells

[2] A short sequence from this film was shown at the Conference. I am grateful to the authors for permission to show the film.

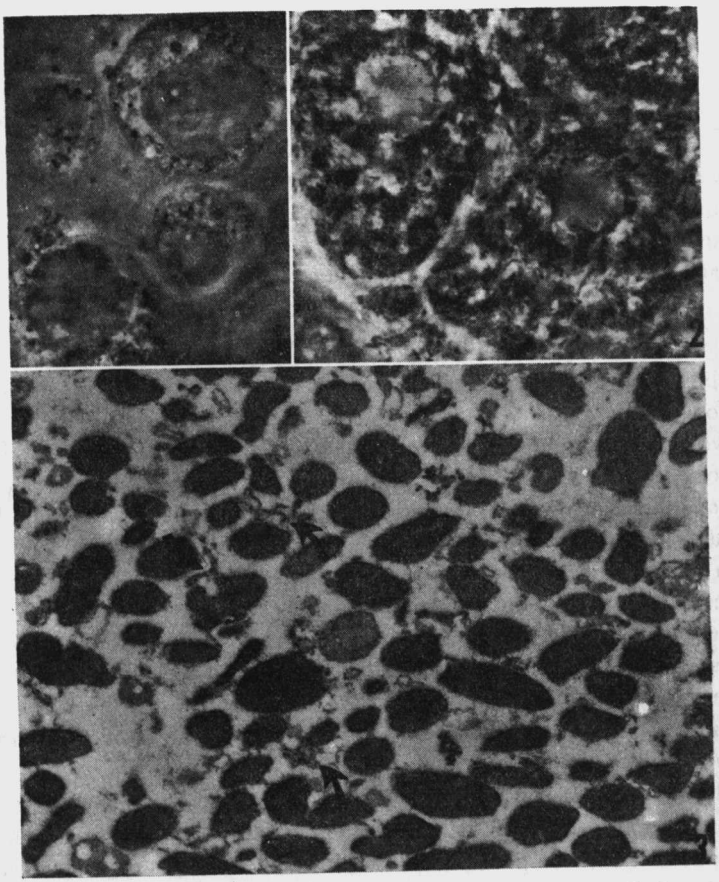

FIG. 1. Phase-contrast photomicrograph of transplantable liver tumor cells in a polyvinylpyrollidone-sucrose homogenate. [From (29).] The cells, somewhat flattened, show large clear nuclei, small elongate mitochondria, and spherical lipid droplets. Objects in focus appear dark or gray, those out of focus are bright. × 950.

FIG. 2. Phase-contrast photomicrograph of parenchymatous cells of rat liver in a polyvinylpyrollidone-sucrose homogenate. [From (29).] The cytoplasm contains many large elongate mitochondria. × 925.

FIG. 3. Electron micrograph of section of mitochondrial fraction. This is an unwashed fraction obtained from a 0.88 M sucrose homogenate of rat liver. The mitochondria are well preserved; their cristae appear as lighter areas. Microsomal contamination is shown by arrows. × 12,500.

of the transplantable liver tumor (fig. 1), they are smaller and less numerous. The striking difference in nucleus to cytoplasm ratio is evident, as is the large number of lipid droplets in the tumor cell. In favorable material, phase contrast microscopy can reveal the presence of structures at times considered artifacts by some. Thus, the Golgi apparatus can readily be seen in these transplantable liver tumor cells (29) and the endoplasmic reticulum was seen by Fawcett and Ito (11) in guinea pig spermatocytes.

Naturally, such purely morphological observations, together with the resolution limitation of light microscopy, leave much room for speculation. The observations do not tell us what really happens when the mitochondria move up to the nuclear membrane, or what the nature and fate of the pinocytosis vacuoles are.

ELECTRON MICROSCOPY

The limitation of resolution may be overcome, to a great degree, by electron microscopy.

Elsewhere in this volume (38) we see new examples of the power of electron microscopy from Dr. Palade, the man to whom not only biochemistry but all of biology and medicine owes so great a debt. I am confident that he, Dr. Keith Porter and other electron microscopists will promptly agree that our dependence upon osmium fixation leaves some important questions unanswered for the moment; for example, that not too much chemical meaning can yet be attached to detailed appearance and precise measurements of membranes in electron micrographs. From the work of Fernández-Morán and Finean (12), of Birbeck and Mercer (4), of Peachey (41), and of Ito and Fawcett (18), it is apparent that the double membranes seen in osmium-fixed sections may result from films of very different biochemical substances.

Yet electron microscopy of thin sections has helped answer many questions of biochemical cytology. Perhaps more important, it has raised many other questions which could not even be phrased without this new look into the near-molecular level within the cell.

To cite a few examples, it is now possible to assert categorically that organelles like the mitochondria of liver survive homogenization and centrifugation quite handsomely, and to indicate the kinds of changes which occur in other subcellular particles.

Figure 3 is chosen because it is sometimes said that mitochondria isolated from liver homogenates in unfortified sucrose solutions do not show good preservation of fine structure. Sections like this show that such is not the case. Mitochondrial fine structure is excellently preserved in this pellet obtained from a 0.88 M sucrose homogenate. The figure also demonstrates how readily contamination by microsomes may be identified. Since microsomes arise largely through fragmentation of ergastoplasm, the ribonucleoprotein granules of Palade (37) and endoplasmic

reticulum of Porter and Palade (42) may be used as identifying markers (27, 28).

Figure 4 is a section through a microsome fraction from the same homogenate. The manner in which the ergastoplasm is fragmented is not yet understood. Generally, the fragments are quite short, as in this figure. Sometimes, as in the so-called 'fluffy layer,' they may be surprisingly long (fig. 5).

That the microsome fraction, even in liver, contains other cytological entities is well recognized. In our laboratory, we are trying to determine the extent to which the so-called 'smooth membranes' seen in the microsome fraction are derived from the microvilli on the surface of the liver cell. These microvilli may be of considerable enzymatic importance, as we shall see later, and they are extremely numerous.

Figure 6 is a section of liver demonstrating the microvilli on the cell surface exposed to the blood sinusoid (space of Disse). The area thus exposed to the blood (plasma?) is very large, and it is covered by literally millions of these tiny extensions of the plasma membrane. We do not know how many microvilli survive homogenization and how many are centrifuged into the microsome fraction.

Figure 6 also shows numerous structures near the sinusoidal surfaces which resemble pinocytosis vacuoles. Such vacuoles are seen in electron micrographs of other tissues and appear most numerous in capillary endothelium (25).

Microvilli are also present over other areas of the hepatic cell, those exposed to the bile canaliculi (fig. 7). As we suggest later, the enzymatic nature of these microvilli may differ from that of Disse space microvilli. Some of the canalicular microvilli sediment into the nuclear fraction, as part of the bile canaliculus fragments (33, 34). It would not surprise us if others end in the microsome fraction.

The section shown in figure 7 passes through a structure with a long, stormy history, the Golgi apparatus. The reality of this organelle can no longer reasonably be questioned, thanks to electron microscopy. Elsewhere in this volume, Dr. Kuff (21) discusses some chemical properties of the Golgi apparatus isolated from epididymis. An excellent review of the Golgi apparatus, by Dr. Palay, has just appeared (39).

From electron micrographs it is difficult to sense what is readily apparent in the thicker sections used for light microscopy, namely that in liver parenchymatous cells the Golgi apparatus is a multiple structure, arranged on either side of the bile canaliculi along their entire length (fig. 9; also (34)).

Figure 7 also shows the peribiliary 'dense bodies,' structures of current interest because they *may* be the lysosomes postulated by de Duve and colleagues. Dr. de Duve refers to the 'dense bodies' when he discusses this important concept of lysosomes later in this volume (8).

Because electron microscopists frequently try to reconstruct processes from static photographs, and because everything is yet so novel, there is a great tempta-

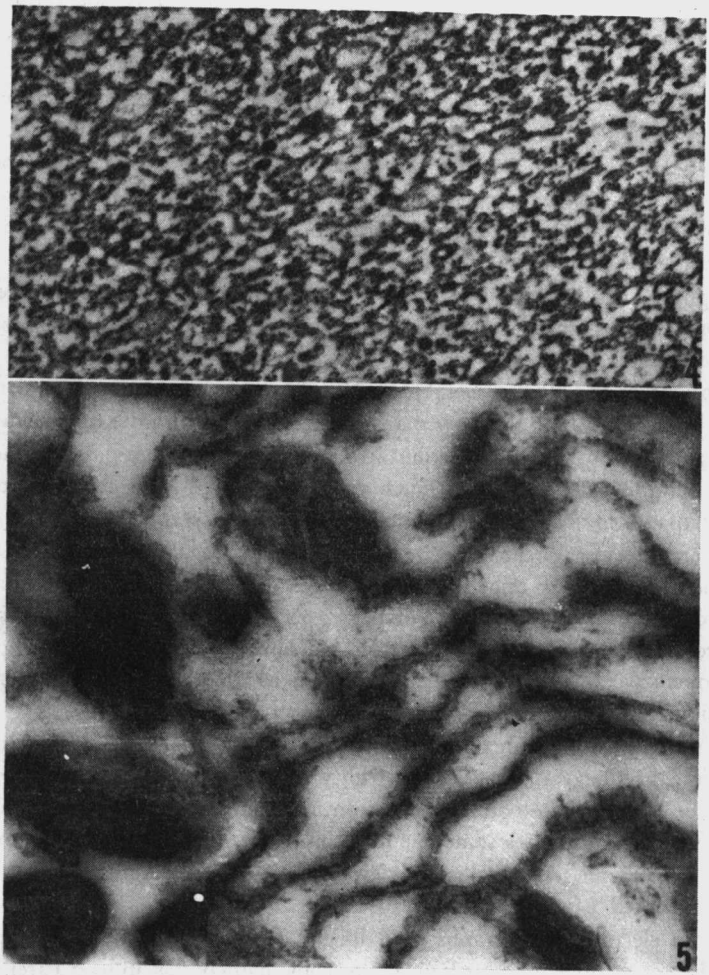

FIG. 4. Electron micrograph of section of microsomal fraction. The bulk of the fraction is made of fragments of ergastoplasm. The ribonucleoprotein granules are readily identified. × 12,800.

FIG. 5. Electron micrograph of section of purified 'fluffy layer' (28). Note long lengths of ergastoplasm, in which both endoplasmic reticulum membranes and ribonucleoprotein granules are readily identified. Mitochondria are fairly well preserved. × 34,250.

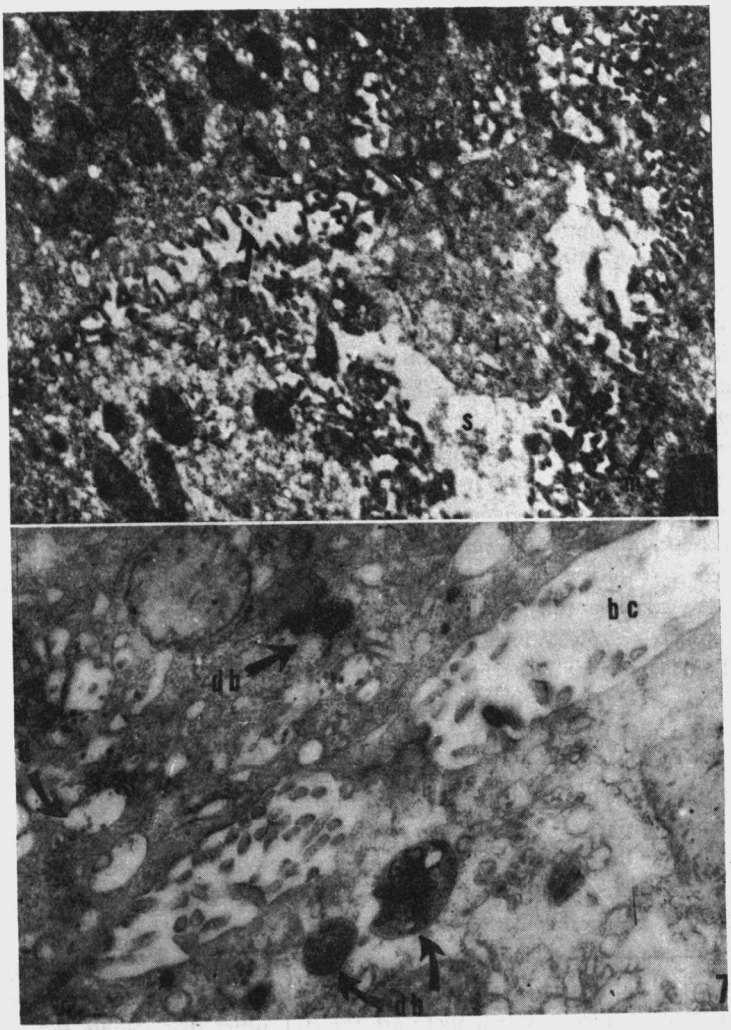

FIG. 6. Electron micrograph of section of rat liver. Shows blood sinusoid (*s*); a portion of a lining cell (*l*), probably a Kupffer cell; and numerous extensions of the parenchymatous cells into microvilli exposed to the sinusoid (space of Disse). Some of the microvilli (*m*) show linearly-arranged granules, producing an apparent cross-striation. Within the parenchymatous cells, note the vacuoles (*v*), dark mitochondria, and the ribonucleoprotein granules of the ergastoplasm. × 10,500.

FIG. 7. Electron micrograph of rat liver. [From (32).] Shows microvilli extending into bile canaliculus (*bc*), peribiliary 'dense bodies' (*db*), and Golgi apparatus (*g*). × 14,400.

tion for speculation; so much so that Sjöstrand, in a recent review, pleads that electron microscopists guard against making the field a 'science of guesses' (48).

However one may feel about such speculation, it is apparent that at best electron microscopy alone remains purely descriptive and essentially morphological. It needs to be supplemented by techniques providing biochemical information.

RADIOAUTOGRAPHY

The most direct approach to *in vivo* biochemical events within particulates is that of radioautography. Dr. Taylor, in this volume, shows the impressive cytological resolution now possible with this technique (54). I will refer to only two reports in the literature.

The first is that of the now classic experiments of Goldstein and Plaut (15). These combine radioautography with the microdissection of living cells possible with *Ameba* and other large cells.

By feeding *Ameba* microorganisms reared on P^{32}-orthophosphate, its nucleus is made to incorporate P^{32} into its ribonucleic acid (RNA). The fate of this radioactive nuclear RNA can then be followed when transplanted into an enucleated half of a nonradioactive *Ameba.* After some 62 hours, considerable radioactivity has moved into the cytoplasm. That this is not a random loss from the nucleus is shown by transplanting a radioactive ('hot') nucleus into a whole nonradioactive ('cold') *Ameba.* While much radioactivity moves into the cytoplasm of the 'cold' *Ameba,* none goes into the 'cold' nucleus.

From indirect evidence, such as effects of ribonuclease digestion, the conclusion is drawn that the material moving from nucleus to cytoplasm is RNA or a material much like it.

Radioautography has another important aspect. It lends itself to fairly good quantitation, as may be illustrated by the work of McMaster-Kaye and Taylor (24). From the time curves of the number of reduced grains appearing over cytological structures, the authors concluded that P^{32} is incorporated into nucleolar RNA considerably earlier than into cytoplasmic RNA.

Even radioautography, however, is not free of difficulties. Aside from dependence upon indirect methods for the identification of the substance in which the radioactivity is incorporated, there are the difficulties that soluble radioactive substances are lost in the methods of tissue preparation currently employed, that ignorance of the nature and magnitude of precursor pools may make interpretation difficult, and that incorporation sites need not necessarily be the sites of function.

ISOLATION OF SUBCELLULAR PARTICLES BY CENTRIFUGATION

Despite all its uncertainties and limitations, the isolation of subcellular fractions by centrifugation of homogenates has provided the main foundation of

biochemical cytology (35). The chief characteristic of the newer cytology is the vast body of biochemical data upon which it is based. The last decade has witnessed an ever-accelerating expansion in our knowledge of metabolic pathways. As new reactions are uncovered their distributions among isolated subcellular fractions are plotted, thus providing an important step towards grasping the interrelationships among these biochemical events. It is fitting that the technique of differential centrifugation was introduced by two cytologists, R. R. Bensley and N. H. Hoerr, and then pursued most vigorously by biochemists, A. Claude, W. C. Schneider and G. Hogeboom, C. de Duve and J. Berthet, O. Lindberg and L. Ernster, and many others. The beautiful work described in subsequent chapters of this volume illustrates the usefulness of such isolated fractions for the biochemist.

We have already seen how microscopy, phase contrast and particularly electron microscopy, can help assess the state of preservation of cell organelles throughout this process and how it can indicate the degree of purity of isolated fractions. It helps also to emphasize uncertainties, such as the fate of microvilli and Golgi apparatus of the liver cell, and possible losses which may occur as the endoplasmic reticulum is fragmented. It has long been known that a considerable amount of material is lost from nuclei during their isolation in aqueous media. In this connection, it might be worth commenting that the 'supernatant fluid' data are the most difficult, of all the fraction data, to interpret in terms of the living cell. For example, it would be of utmost significance, in terms of *in vivo* function if not in terms of biochemical sequences, to know that the amino acid-activating enzymes discussed by Dr. Stephenson later in this volume (49) are truly in a soluble phase bathing the ergastoplasm and mitochondria. But I believe we cannot categorically assert this to be the case. There remains a possibility that these, like other enzymes of the 'supernatant fluid,' may have leached from the nucleus, endoplasmic reticulum, or some other organelle.

There is, thus, uncertainty about some localizations indicated in figure 8, even for the liver cell. However, there can be little question that the mitochondria are the chief sites of oxidative phosphorylation in the cell. Dr. Allfrey, later in this volume (2), discusses an adenosine triphosphate (ATP) synthesis by isolated nuclear fractions of thymus; but it remains to be seen to what extent the findings regarding thymus and other lymphoid tissues can be generalized to tissues like liver.

The work of Chance and colleagues has demonstrated that isolated mitochondrial suspensions show the same sequence of events in electron transport as do suspensions of *living* yeast and tumor ascites cells. Chance comments on this important achievement in these words: "It is, of course, of considerable reassurance to biochemists that the isolated material does not involve a serious artifact" (5).

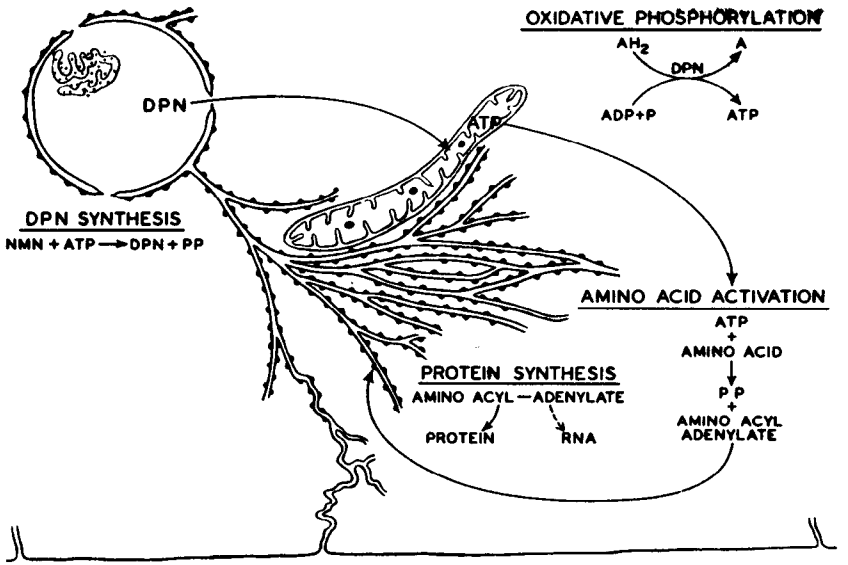

FIG. 8. Schematic representation of complexity and interrelatedness of subcellular units in the rat liver cell. [From (35).] *Abbreviations:* DPN, diphosphopyridine nucleotide; NMN, nicotinamide mononucleotide; ATP, adenosine triphosphate; PP, inorganic pyrophosphate; AH_2, oxidizable substrate; A, oxidized substrate; ADP, adenosine diphosphate; P, orthophosphate; RNA, ribonucleic acid.

The work of Preiss and Handler (44) suggests that perhaps the reaction for DPN synthesis which the diagram (fig. 8) shows, first described by Kornberg (19), may not be the important pathway of diphosphopyridine nucleotide (DPN) synthesis in the cell. However, it is of interest that at least one of the steps of DPN synthesis from free nicotinic acid (44) is also recovered in the nuclear fraction (43).

From the work Dr. Stephenson describes (49) there is little doubt that amino acid incorporation, probably an integral part of protein synthesis, occurs in the microsomal ribonucleoprotein granules, although as Loftfield (22) says in his masterful review, "the final step in forming the protein may be disturbed" in these cell-free systems.

Irrespective of changes which will be required in this diagram, the data from isolated subcellular fractions make the conclusion inescapable that subcellular particles are interdependent metabolically. The diagram does not attempt to show interrelationships among nucleotides, RNA and DNA (deoxyribonucleic acid). Nor does it show lysosomes, dense bodies and other complexities of the liver cell. These are shown diagrammatically in figure 9; this, too, omits some known structures and greatly oversimplifies others. It is based not only upon

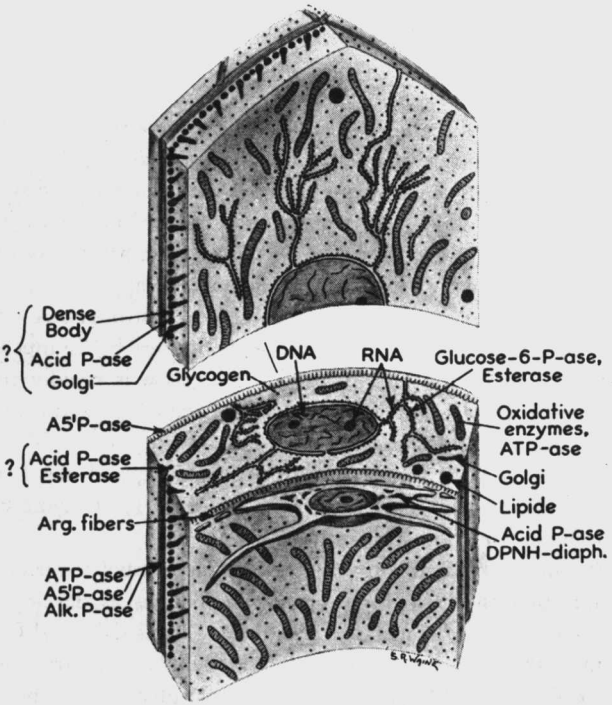

FIG. 9. Schematic representation of a parenchymatous cell of rat liver, with adjacent Kupffer cell (stellate shape). Note orientation of mitochondria (long axes oriented in sinusoid-canaliculi directions). The pericanalicular arrangement of Golgi apparatus, dense bodies, acid phosphatase bodies and esterase bodies is shown; the uncertainty concerning their interrelations is indicated by question marks. *Abbreviations:* Acid P-ase, acid phosphatase; A5′P-ase, 5′-nucleotidase; ATP-ase, adenosine triphosphatase; Alk. P-ase, alkaline phosphatase; Glucose-6-P-ase, glucose-6-phosphatase; DPNH-diaph, DPNH-tetrazolium reductase; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; Arg. fibers, argyrophylic fibers.

biochemical assay of isolated fractions and electron microscopy, but also on the results of specific staining methods.

SPECIFIC CYTOCHEMICAL STAINING METHODS

We will refer to only one cytochemical staining method for nonenzymatic constituents, the Feulgen method for DNA (for a fuller discussion of this and other methods see refs. 26, 30). It has recently been applied to a problem which may be of considerable interest to *in vivo* function. Even if it prove of less physiological interest than now appears, the work illustrates the possibilities of quantitation with some of these staining methods. It is agreed by most that, with but few exceptions, the DNA content of cells is generally constant. Yet, at least