A Short History of Tissue Fractionation

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The period immediately following the end of World War II will be remembered in the history of cell biology as that of the great breakthrough. As with many scientific advances, new tools, not new thoughts, rendered possible the massive invasion of the subcellular world that was launched at that time. The availability of the electron microscope, and the development of procedures allowing the examination of biological samples with this instrument, made the cell accessible to detailed morphological exploration. At the same time, the introduction of chromatography, of radioisotopes, and of spectrophotometers and other refined physical instruments, enhanced enormously the power and incisiveness of biochemical analysis.

Revolutionary as these developments were, they would, nevertheless, not have sufficed in themselves for the construction of a true cell biology. What was needed, in addition, was a bridge between morphology and biochemistry, a junction between the essentially parallel avenues opened by these two disciplines, a hybrid methodology whereby the visible and the measurable could be correlated into a unified picture of the living cell. Tissue fractionation provided this indispensable link.

Like many important scientific advances, tissue fractionation owes its development to the vision and effort of a few innovators. It is, however, very far from being a monolithic construction, built according to carefully conceived plans. Rather it has arisen in a somewhat haphazard and untidy fashion from the meshing together, sometimes intended, sometimes accidental, of a remarkable diversity of interests. Physical chemists, engineers, biochemists, virologists, molecular biologists, have all had their input, in addition to the cytologists themselves. Their combined contributions have produced a vigorous and highly successful hybrid, which nevertheless still betrays traces of its mixed parentage in the uncertainties that surround some of its concepts and applications. Part of this haziness is due also to the nature of the object of tissue fractionation. Any reductionist approach to the complexity of the living cell must perforce proceed by successive approximation. This kind of progress is very apparent from the crude fractionations of yesteryear to the sophisticated dissections that are being carried out in many laboratories today. Clearly, the process of growth is still con-

tinuing, and we must be prepared to revise our rules and to refine our methods as our understanding of cellular organization becomes deeper and more detailed.

In writing this chapter, we have made no attempt to provide a comprehensive review of the whole field. This would have been quite impossible, in any case, within the space made available to us. On the assumption that our readers will be more familiar with the present than with the past, we have chosen to dwell mostly on the early history of tissue fractionation. It is our hope that we may in this way give the young generation of cell biologists a certain feel for the manner in which what is now textbook knowledge was actually uncovered, reminding them at the same time of certain fundamental principles which still remain true today, even though they were laid out some 30 years ago.

Broadly speaking, the history of tissue fractionation can be divided into three parts. First, there is the long period of germination, much of it subterranean and not recognized as such until later, ending with the publication of the two historical papers by Albert Claude in 1946 (1, 2). Then comes a period of explosive growth and luxurious blossoming, which more or less terminates with the untimely death of George Hogeboom in 1956. After that date, the pace quietens again, and we enter into an era of slow, progressive maturation, which is still continuing.

Early Preparative Attempts

Friedrich Miescher is generally given the credit for having first used a centrifuge to isolate a cell organelle. In 1869, he separated nuclei from human pus cells stripped of cytoplasm by peptic digestion (3). His techniques were crude, and his preparations would hardly pass muster today. But the outcome turned out to be a major one because it resulted in the discovery of a new class of biological constituents, which Miescher called "nucleins" as a reminder of their nuclear origin, and Altmann renamed "nucleic acids" when their acidic character was recognized.

Since these historical experiments, the isolation of pure nuclei has been pursued by numerous investigators using a variety of techniques. Unfortunately, such attempts were long hampered by the occurrence of an unknown degree of cytoplasmic contamination, which could be neither avoided nor properly assessed. Consequently, most of the analytical work performed on isolated nuclei until the early 1950s, especially that relating to enzyme activities, is practically valueless today. As a matter of fact, much remains to be known concerning the

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enzymic equipment of nuclei, and opinions are still divided on the extent to which soluble proteins may be lost by diffusion through the nuclear envelope in the course of the isolation of these organelles (4, 5).

This difficulty was recognized at an early stage by Martin Behrens, who devoted a long series of painstaking investigations to the isolation of nuclei from powdered lyophilized quick-frozen tissues fractionated in nonaqueous media (6). Although his procedure was later adopted in the laboratory of Alfred Mirsky (7), it never gained wide acceptance. The damage inflicted on cell constituents by freezing and by exposure to organic solvents is generally considered prohibitive. As a means of keeping ions and small molecules trapped inside cell organelles during isolation, quick-freezing and fractionation in nonaqueous media will, however, continue to serve a limited, but indispensable use (4, 5). The work of Behrens also deserves to be recalled for another reason. He was the first to separate cell components by equilibration in a density gradient, a technique which, transposed to aqueous media, became a major fractionation tool 20 years later.

Other landmarks in the history of tissue fractionation are the attempts made in the laboratory of R. R. Bensley to separate and to analyze pure mitochondria (8), and the isolation of microsomes by Claude (9). It is interesting that the latter discovery concerned a cell component that could not be seen as such in the light microscope and was, therefore, not known to exist (except in those cells where it forms large basophilic masses, or ergastoplasm). Claude's aim was actually to purify the agent of the Rous sarcoma, and he discovered the microsomes in the course of a control run carried out on uninfected chick embryos.

An important prewar development, without which tissue fractionation would perforce have remained very crude and incomplete, was the construction of more powerful centrifuges. The great pioneer in this domain was the Swedish scientist, The Svedberg, who not only built the first high-speed centrifuges, but also worked out a detailed theory of the behavior of macromolecules subjected to a centrifugal field and applied it to the measurement of molecular weights. Reviewed in the masterful treatise by Svedberg and Pedersen (10), this work became an inspiring source of information to those who later attempted to move centrifugal fractionation from the empirical to a more scientific basis.

Svedberg's ultracentrifuge, however, was essentially an analytical instrument, specifically designed for the accurate recording of sedimentation boundaries. Its conversion for preparative use would have been impossible, for the simple reason that its rotor axis was horizontal. It rested on bearings that were lubricated and cooled by circulating cold oil and was driven by two oil turbines situated at each end of the shaft. Heat exchange from the rotor was facilitated by a stream of hydrogen. The sample cell had transparent windows, which allowed the continuous observation of the sedimentation boundary while the centrifuge was running.

The transition from this analytical instrument to modern preparative ultracentrifuges came in very indirect fashion through the efforts of Emile Henriot, a French physicist established in Belgium, who, independently of Svedberg, had been able to achieve very high rotational speeds by means of a bearingless top, driven and supported at the same time by compressed air (11). The uses of this machine remained, however, limited, until Jesse Beams and Edward Pickels, in the United States, conceived the idea of adapting it to drive a larger rotor suspended by a steel wire (12). This device had the additional advantage that it permitted the rotor to be maintained in a vacuum chamber, thereby reducing considerably the braking and heating effects of frictional forces.

This new centrifuge still served only an analytical purpose. What prompted its further transformation into a preparative instrument was a demand from investigators interested in the isolation of viruses. This demand brought Pickels from the University of Virginia to the laboratories of the International Health Division of The Rockefeller Foundation, which at that time was located at The Rockefeller Institute for Medical Research in New York (13). There, with Johannes Bauer, Pickels built the first "high speed vacuum centrifuge suitable for the study of filterable viruses" (14), essentially a modification of the air-driven analytical centrifuge of Beams and Pickels (12). Later, he went on to develop a much more convenient electrically driven ultracentrifuge (15).

It is of some historical interest that these important technical developments seem to have had little influence on the efforts that were made, at the same time and literally under the same roof, to adapt centrifugation to quantitative tissue fractionation. Apparently, the Pickels machines were not used by Claude (1, 2, 9), nor by his immediate pupils (16) at The Rockefeller Institute, who relied for all their high-speed centrifugations on the so-called multispeed attachment of the International centrifuge. This was a rather primitive device, which allowed a small angle-head to be driven at up to 18,000 rpm by a belt-and-pulley connection with the main centrifuge shaft. It developed a maximum field of about 18,000 g at the bottom of the tubes. it also developed a considerable amount of heat, its temperature rising to 15°C in an ordinary cold room (1), and to 10°C in a −5°C cold room (17), during the 90-minute runs needed for the isolation (incomplete!) of the microsomal fraction.

Eventually, however, the two lines of research converged, paradoxically when Pickels left The Rockefeller Institute to found Specialized Instruments, Inc., in Belmont, California, whence came the famous Spinco analytical Model E, and preparative Model L, ultracentrifuges. Those who were engaged in cell fractionation at that time will never forget their sense of wonder and delight, almost of reverence, when they first unpacked the slick new instrument which completely changed their lives.

One last landmark deserves to be recalled, seemingly humble, but of immense practical importance, namely the construction by Carl Ten Broeck (18), of a hand-operated coaxial tissue homogenizer, which served as model for the motor-driven device later developed by Potter and Elvehjem (19), now in universal use. In fact, the credit for this discovery, as was pointed out by Potter himself (20), should really go to W. A. Hagan, who designed a very similar instrument in 1922, at a time when its utility was not yet perceived (21). Be this as it may, there is no doubt that popularization of the Potter-Elvehjem device served for the first time to inculcate in biochemists some measure of respect for biological structures. The standard procedure in those days was to release every cell constituent that could be made soluble by the whirling blades of a Waring blender, and then to throw the insoluble "residue" down the drain.

Development of Quantitative Analytical Fractionation: The Turning Point

Such, approximately, was the state of the art in the early 1940s. The idea that cell organelles could be separated for

analysis by centrifugal methods was obviously shared by a number of leading cytologists, and several attempts in this direction had already been carried out. Furthermore, improved instruments that could be used successfully for this purpose were becoming available. But something was still missing, namely the *analytical approach*. As exposed elsewhere (22), the development of this approach represents Claude's most important contribution. The point is a subtle one and deserves to be reemphasized.

Until then, investigators had followed mostly a preparative approach, trying to answer the question: WHAT IS IN . . . this or that subcellular entity that can be seen in the microscope? The question is, of course, a valid one, and the logical way of trying to answer it is by purification of the object of interest, followed by analysis. Unfortunately, centrifugal separation methods, especially those that were available 40 years ago, are really very crude. On the other hand, most subcellular organelles are, for a variety of reasons that need not be gone into here (see, for instance, references 22–24), very difficult to isolate in good yield and in a satisfactory state of purity and integrity. Compounding these difficulties was the fact that there is no way of ascertaining the purity of a subcellular preparation without the help of an electron microscope and/or of appropriate biochemical markers, both of which were unavailable at that time. Therefore, the preparative approach was bound to give equivocal results, as it did, for instance, in the case of nuclei.

What Claude did, when he enlisted the collaboration of Rollin Hotchkiss and of George Hogeboom in the investigation of the enzyme content of his subcellular fractions, was to ask a new kind of question, namely: WHERE IS . . . this or that enzyme that can be measured in the test tube? To answer such a question, a new approach had to be developed, in which "special emphasis was attached to the quantitative aspects of the results and efforts were made, whenever possible, to express the enzymatic activity exhibited by each fraction in terms of the total activity possessed by the unfractionated liver extract" (25). These were the guiding principles that led Claude to develop the *quantitative* fractionation procedure that is described in detail in the two seminal papers he published in 1946 in *The Journal of Experimental Medicine* (1, 2). The analytical approach, much more than the technical details—these were, in fact, never adopted by anyone—represents the essential contribution of these papers.

Indeed, less than two years after it was first described, Claude's method was largely reshaped by his young collaborators, George Hogeboom, Walter Schneider, and George Palade (16). For incomplete grinding by gently "rubbing the cells against each other" with a mortar and pestle, they substituted quantitative cell breakage by means of a Potter-Elvehjem homogenizer. This allowed separation of a nuclear fraction—discarded with unbroken cells and gross debris in Claude's original procedure—and of a more representative cytoplasmic fraction. Instead of "physiological" saline, they used hypertonic (0.88 M) sucrose as medium, which minimized agglutination of particles and also preserved the elongated shape of mitochondria. Thanks to this property, and with the additional help of Janus green staining, the workers were also able to identify Claude's "large granules" decisively as consisting mostly of mitochondria. The high viscosity and density of hypertonic sucrose did, however, greatly complicate and prolong the manipulations, and Schneider (26) soon published an alternative procedure using 0.25 M sucrose, which became widely adopted.

The years that followed publication of these historic papers witnessed a remarkable flourishing of enzyme distribution studies. The four-fraction scheme was rapidly adopted by the biochemical community, and soon became part of the regular enzymological arsenal. The findings obtained generated tremendous excitement. Meeting upon meeting was organized around the topic. Chapters and reviews were written almost every year. Heated discussions opposed the tenets of different doctrines, each side accusing the other of "artifact." In short, the biological world was in ferment, having realized—quite rightly—that history was being made.

In this crucial period, Hogeboom emerged as a major leader. In collaboration with Schneider, he investigated the distribution of numerous enzymes, keeping track at the same time of the results of others, which he surveyed comprehensively and critically in a number of reviews. Later, with Edward Kuff, he pioneered the use of density gradient centrifugation, and showed that this technique could be used both for the determination of molecular weights in crude solutions and for the analytical fractionation of subcellular particles. Especially, he championed relentlessly the proper adherence to Claude's analytical approach, insisting, sometimes in the face of severe opposition, on the "need of establishing balance sheets in which the summation of the activities of the tissue fractions is compared with that of the whole tissue" (27), and drawing attention time and again to the erroneous interpretations that were introduced into the literature as a result of poor techniques and sloppy thinking. His death in 1956, at the early age of 43, was an irreparable loss to cell biology. The second volume of *The Journal of Biophysical and Biochemical Cytology* is dedicated to his memory. His biography and list of publications, which appeared in that issue, are recommended reading to anyone interested in tissue fractionation (28).

By the time of Hogeboom's death, tissue fractionation had definitely come of age. It was used in many laboratories, and a certain consensus had emerged on methodology and nomenclature. As witnessed by a number of contemporary reviews (27, 29–33), fractionation of homogenates into "nuclei," "mitochondria," "microsomes," and "supernatant" was almost universally adopted. Even when workers departed from this scheme, they tended to discuss their results in the framework of an essentially dual division of cytoplasmic particles (34, 35). The danger of doing so was recognized by some investigators, for instance by Van Potter, who wrote in 1951: "The misuse of the words nuclei, mitochondria and, microsomes is . . . regrettable, although all of us are guilty in varying degrees" (36).

How treacherous this trap actually could be was revealed to us at an early stage in some experiments that were undertaken in 1949 by our group at the University of Louvain in Belgium. Attracted to tissue fractionation by the apparent insolubility of the hepatic enzyme glucose-6-phosphatase, we made two sets of observations that turned a passing interest in the methodology into a lasting commitment. The first one was that glucose-6-phosphatase comes down largely with the microsomal fraction and most likely is associated exclusively with microsomal particles, to which it is firmly attached (17). This finding, together with the similar conclusion reached by Hogeboom et al. (37) with respect to the exclusively mitochondrial localization of cytochrome oxidase, opened the way to the use of these enzymes as *markers* of their host organelles. Furthermore, the heterogeneity of their distribution, unless stringent precautions were taken, emphasized for us the important distinction between a cell constituent and the subcellular fraction in which it is concentrated.

Our second observation concerned acid phosphatase. First, we found that in freshly prepared homogenates this enzyme occurs largely in a latent, sedimentable form, which biochemical experiments helped to characterize as a saclike particle containing the enzyme within the confines of a substrate-impermeable membrane. Further, we found the particle-bound acid phosphatase to be distributed between the sedimentable fractions in a manner that differentiated it clearly both from the mitochondrial cytochrome oxidase and from the microsomal glucose-6-phosphatase. These findings prompted the development of a new fractionation scheme (38), which, with the help of the marker-enzyme hypothesis, eventually led to the recognition and characterization of lysosomes, and later of peroxisomes, as components of the mitochondrial fraction (22, 23, 39, 40).

Density Gradient Centrifugation and Other Refinements

An important development of the early 1950s was the introduction of density gradient centrifugation into the tissue fractionation arsenal. It is of some interest that here again, the first impetus came from virus research. The pioneer of the technique was a plant virologist, Myron K. Brakke, working at the Brooklyn Botanic Garden (41, 42). He developed zonal sedimentation through a sucrose gradient for the separation of potato yellow-dwarf virus, using a rotor that we are told in a footnote was designed on special order by Josef Blum (the head of The Rockefeller Institute instrument shop), and built by Ivan Sorvall, Inc.

The first application of Brakke's method to tissue fractionation was made by Heinz Holter and his co-workers at the Carlsberg Institute in Copenhagen (43). Thanks to the availability of the newly developed Spinco SW-39 swinging-bucket rotor, capable of withstanding fields as high as 165,000 g at the bottom of the tube, they were able to bring both mitochondria and microsomes close to density equilibrium in a sucrose-"Diodon" gradient. Diodon, a densely iodinated solute, was incorporated in the sucrose gradient in order to allow the required densities to be reached in media of lower viscosity and osmotic pressure than can be done with sucrose alone. This example was not followed, and most workers subsequently went on with Kuff and Schneider (44) to use sucrose alone for the construction of gradients. The principle of the Diodon method has, however, been revived recently by the introduction of Metrizamide (an iodinated carbohydrate derivative made by Nyegaard and Co. A.S., Oslo, Norway).

A different application of Brakke's technique was developed at the Argonne National Laboratory by John Thomson and collaborators (45–47), based, as in Brakke's own virus work, on incomplete zonal sedimentation through a stabilizing density gradient. By introducing some simplifying assumptions into Svedberg's equation, these workers were able to derive the approximate size of the sedimenting particles from the position reached in the gradient by their associated enzymes. The same type of information was obtained by Kuff et al. (48) in yet another form of density gradient centrifugation, first employed by Kahler and Lloyd (49) to analyze the sedimentation of polystyrene latex particles, by using a swinging tube rotor of their own design. In this method, the sample subjected to analysis, instead of being layered above the gradient, is actually incorporated homogeneously in it, and the appropriate size distribution of the sedimenting particles is derived from the position and shape of their sedimentation boundary, as in

conventional analytical centrifugation. An advantage of this technique is that it is not subjected to artifacts due to "drop sedimentation," a well-known phenomenon whose importance was pointed out by Anderson (31).

Biochemists were quick to appreciate the power of density gradient centrifugation in preparative rotors, which extended vastly the domain of applicability of analytical ultracentrifugation. After the first attempts by Hogeboom and Kuff (50), and the refinement of their technique by Martin and Ames (51), preparative centrifugation became widely used for the determination of the molecular weight of enzymes and of other materials that could be measured by specific methods. Zonal sedimentation is now part of the standard arsenal of molecular biology, especially in the analysis of nucleic acids and ribosomal preparations. A particularly elegant application of isopycnic centrifugation is that developed by Meselson, Stahl, and Vinograd (52), who used self-generating gradients of cesium chloride to separate DNA species of different density. The demonstration of semiconservative replication by Meselson and Stahl (53) was an early triumph of this technique.

In many such applications, the fact that sedimentation occurs through a density gradient, rather than in a homogeneous medium, introduces relatively minor complications. This is not so, however, in the case of tissue fractionation. When membrane-bounded subcellular particles move through a gradient of sucrose or of some other low molecular-weight solute, their density increases progressively, owing to permeation of solute, or to osmotic loss of water, or to both phenomena occurring simultaneously. Because sedimentation is a function of the difference between the density of the particle and that of the medium, the continuous increase in density suffered by the sedimenting particles causes them to move far beyond the level in the gradient corresponding to their normal density, and to become arrested only at a final equilibrium position, which is a complex function of their physical characteristics. In addition to the injuries dehydration may inflict on the particles, this phenomenon forces them to traverse layers of such high viscosity that the centrifugation time may become prohibitively long. Increasing the centrifugal field, as has become possible with modern titanium rotors, has not proved as helpful in resolving the latter problem as might have been hoped, because of another complication. As first shown by Wattiaux et al. (54), and directly confirmed by Bronfman and Beaufay (55) with a high-pressure chamber, particles may undergo extensive damage when subjected to the high hydrostatic pressures that are generated in high-speed rotors.

Searching for a solute that would avoid some of these difficulties, Holter and Max Møller (56) first tried a number of commercially available macromolecules, and finally had one made to order, according to the following description: "sufficiently high molecular weight to insure low osmotic activity even in highly concentrated solutions, sphaerical molecules to insure low viscosity, high solubility in water and weak salt solution, chemical inertness and stability to autoclaving, lack of toxicity, absence of nitrogen (so as not to prevent Kjeldahl analysis)—and finally, as high specific gravity as possible." A substance meeting most of these specifications was synthesized in the Swedish firm Pharmacia by B. Ingelman and P. Flodin, and marketed under the name "Ficoll." It has one additional advantageous property, which was not included in the order: with a molecular weight of only 50,000, it does not have the high sedimentation coefficient that complicates the use of many other macromolecules.

We encountered the latter problem in our own use of glycogen as a solute (57). Nevertheless, we were able to investigate experimentally the effect of sucrose concentration on particle density by means of isopycnic centrifugation in isosmotic gradients of glycogen made up with sucrose solutions of different concentrations (57). The results obtained in these experiments, which also included a number of trials in gradients made in D_2O, to assess particle hydration, were fitted to a theoretical model (58, 59). From this analysis, estimates could be obtained for a number of typical properties of rat-liver mitochondria, lysosomes, and peroxisomes, including: density and hydration of the particle matrix, size of the sucrose-accessible space, and content in osmotically active solutes (39, 57, 59). Once the dependence of particle density on sucrose concentration was known, the distribution of particle size could be derived accurately from the analysis of sedimentation boundaries (60) or of sedimenting zones (61). Results obtained in this manner were found to be in good agreement with those of other approaches, including quantitative morphometry (62, 63).

As has been pointed out repeatedly elsewhere (22, 23, 39), such experiments are nothing but an extension of the original Svedberg analytical approach from the macromolecular to the submicroscopic, and even microscopic, range. They do, however, depend on the availability of some method for the quantitative evaluation of the particles in the subfractions separated from the gradient at the end of the experiment. Unless the particles have been first purified, which is rarely the case, one requires for this purpose a characteristic biochemical constituent, usually an enzyme, uniquely located in the particles under consideration (postulate of unique location). In addition, the relation of this constituent to the total particle mass or protein content must be known, or some such relation must be assumed. The simplest assumption is that the content of a particle in a specific biochemical marker is proportional to particle mass or protein content, which is another way of saying that all the particles in a given population have the same biochemical composition, irrespective of size (postulate of biochemical homogeneity). Within the limits of present experimental techniques, both postulates have been verified reasonably well for several markers, thus serving their purpose (64, 65). But this does not mean that the marker-enzyme hypothesis should be in any way generalized, or extended beyond its purely operational limits. It never was, and exceptions to its underlying postulates were recognized from the very beginning of its application (38). The fact that it does apply to a number of membrane-bound enzymes is, however, remarkable, and raises interesting problems of subcellular organization. These will be alluded to at the end of this chapter.

The increasing interest in density gradient centrifugation has also spurred a great deal of instrumentation research. The pioneer in this domain is undoubtedly Norman Anderson, who, as early as 1955, started experimenting with a variety of devices designed to avoid or to minimize the artifacts that are caused by wall effects, drop sedimentation, mechanical convections during acceleration and deceleration of the centrifuge, and other disturbing phenomena inevitably associated with the operation of swinging-bucket rotors (66–68). Thanks to the availability of the unique engineering facilities of the Oak Ridge National Laboratory, his efforts have culminated in the development of a number of entirely automatic zonal centrifuges, of which several are now produced commercially (69).

In our laboratory, the main efforts have been devoted to the construction of automatic rotors of a different design (70) that combine a high efficiency with a low hydrostatic pressure, and are particularly well suited, therefore, for isopycnic centrifugation. Much ingenuity also has been expended in the development of various accessories, such as gradient makers, sampling devices, rotor stabilizers, graded-speed accelerators and decelerators, centrifugal field integrators, etc. (see, for instance, references 31, 58, 69). Commercial firms have joined in this effort, and workers now have available a rich choice of instruments that represent the technological returns of tissue fractionation.

Another key advance of the late 1950s was the development and generalization of techniques for the examination of subcellular fractions in the electron microscope (48, 71). These were eventually complemented by the introduction of quantitative morphometric procedures (62, 63). It thus became possible to confront directly the biochemical and the morphological properties of subcellular fractions.

Thanks to these various developments, the progressive dissection and biochemical characterization of the different parts of rodent-liver cells, first initiated by Albert Claude, has now been extended to most morphologically recognizable cell components, and a large number of other tissues and cell types have been similarly investigated. As already mentioned, Hogeboom et al. (16) found that Claude's "large granules" consist mostly of mitochondria. Later, the occurrence of nonmitochondrial particles as "contaminants" of this fraction was recognized in our laboratory. This work led to the biochemical characterization of lysosomes (38) and of peroxisomes (57, 72), and allowed the morphological identification of the former as the "pericanalicular dense bodies" (73, 74), of the latter as the "microbodies" (74), described by electron microscopists. The mitochondria themselves were successfully subfractionated by Parsons et al. (75) and by the late Jack Greenawalt and his coworkers (76), allowing the separate biochemical characterization of the outer membrane, the inner membrane, the intermembrane space, and the matrix of these particles.

Other important landmarks were the discovery by Palade and Siekevitz (71, 77) that the microsome fraction is made up largely of vesicles derived from the endoplasmic reticulum, and the subsequent subfractionation of these vesicles into luminal content, membrane constituents, and ribosomes (71, 78–80). Further knowledge about cytomembranes was provided by the development of methods for the isolation of plasma membranes (81, 82) and of Golgi components (83–85), which allowed the identification of characteristic biochemical markers for these cell components, for instance 5'-nucleotidase for plasma membranes (86) and galactosyltransferase for Golgi elements (83, 87, 88). These findings, in turn, have made possible a detailed analytical subfractionation of the microsomal fraction, which has revealed that vesicles derived from the smooth and rough endoplasmic reticulum account for only about 75% of the total microsomal proteins (89). The remainder belongs to plasma membrane fragments, Golgi elements, and a third component, probably originating from torn-off mitochondrial outer membranes, with a minor contribution from larger cytoplasmic particles.

How these and the numerous other discoveries that have arisen from tissue fractionation have opened the way to the elucidation of such fundamental cellular processes as oxidative phosphorylation, intracellular digestion, protein synthesis, bulk transport in and out of cells, secretion, organelle biogenesis, and the underlying metabolic processes and their control, is illustrated by many of the other chapters in this book.

Future Prospects

Today, cell biology has come to display so many exciting vistas, and to offer so many sophisticated tools to explore them with, that primary biochemical mapping of cells by quantitative tissue fractionation has lost some of the glamour and appeal it had 25 years ago. This is understandable, but nonetheless regrettable, because the job is still far from finished. There are many enzymes whose intracellular location is unknown, or is believed to be known, but actually would bear reinvestigation. Easily forgotten in this connection is that much of our knowledge of enzyme localization is really derived from fairly primitive experiments that lack many of the controls that are available today. Fatty acid β-oxidation is a good example. Classically ascribed to mitochondria, this process has now been found to take place in both peroxisomes and mitochondria (90, 91).

Even the best-established data may deserve closer scrutiny. Tissue fractionation remains a relatively gross tool, whatever the sophistication of the techniques used, and it can reveal only the major site or sites of localization of enzymes and other specific biochemical constituents, not minor ones. For these, special methods are needed, usually based on some sort of cytochemical or immunochemical form of visualization. Other difficulties, not to mention artifacts, may, however, complicate the interpretation of the results of such approaches. Lack of quantitation is one; accurate identification of the "decorated" structure is another.

Modern work on cytomembranes serves to highlight these uncertainties. It is becoming increasingly clear that extensive fusion takes place continuously in living cells between membranes belonging to different domains, for instance in the course of secretion, of endocytic uptake, and of other cellular processes requiring bulk transport. Such fusion events should be detectable morphologically by the coincidence of markers of the two domains concerned on the same continuous piece of membrane. Not only should these markers be seen side by side, but they might also be expected to intermingle by lateral diffusion in the plane of the membrane. Considering, in addition, the intensity of this kind of traffic as revealed, for instance, by estimates of membrane recycling, a considerable degree of randomization of markers would appear quite likely, except, of course, that they would not be identified as markers anymore.

In the light of these considerations, the manner in which certain enzymes remain confined to a specific membranous domain is quite remarkable. Presumably some fuzziness occurs at fusion boundaries, but it has not proved important enough to be detectable by fractionation techniques. On the contrary, any improvement in resolution has served only to sharpen the distinction between different domains. This point is illustrated by the results of Amar-Costesec et al. (92), and, even more dramatically, by some recent findings by Ito and Palade (93). By using a specific immunoadsorption method, these workers were able to remove selectively from a highly purified Golgi fraction vesicles containing the endoplasmic reticulum marker glucose-6-phosphatase, from vesicles bearing the typical Golgi marker galactosyltransferase. Interestingly, glucose-6-phosphatase was accompanied by two other enzymes with which it is associated in the endoplasmic reticulum, NADH cytochrome c reductase and NADPH cytochrome c reductase. As a matter of fact, the antibody used in the separation was directed against the NADPH-specific reductase.

This interesting experiment thus demonstrates that even in a purified preparation, where fuzziness might have a better chance of being detectable, it did in fact not show up. Some vesicles in the preparation had the composition of endoplasmic reticulum, as indicated by three enzyme activities, and others that of Golgi membranes, as revealed by at least one enzyme. The authors insist that both vesicles were "reliably identified" as Golgi elements, on the basis of morphological criteria. This, however, raises a semantic problem. What name should one give to a structure that looks like A, but has the chemical composition of B?

This is but one of the many problems that are left for future generations to solve. The role tissue fractionation will play in this continuing adventure may tend to be overshadowed by the numerous new cytochemical tools that have become available. But it would be a great mistake to abandon this valuable approach, which alone can provide the kind of documented and quantitative biochemical information needed both for our knowledge of cellular organization and for the development of many of our cytochemical techniques.

An important lesson of the past 35 years lies in the magnitude of the effort that has gone into the development of better instruments, new materials, and more refined approaches, based on a deeper understanding of the physical parameters involved. This effort should be pursued, for it is as true today as it was 35 years ago that "further advance has to await the accident of technical progress" (94).

It is doubtful, however, that centrifugation alone will provide the new tools that are necessary, for we have come close to the point where distinct components of the cell cannot be separated because they do not differ sufficiently from one another in size and in density. Then, one has to find means of modifying selectively the properties of one component, or one must take advantage of other properties by which the components differ sufficiently to achieve their separation.

Examples of the first approach are the selective changes in density that can be produced in lysosomes by injection of such substances as Triton WR-1339 (95, 96) or dextran (97), which accumulate in these particles; in mitochondria by the nutritional rationing of an essential phospholipid building block (98), or by active uptake of calcium (99); in cholesterol-rich membranes by digitonin binding (92, 100); and in rough endoplasmic reticulum vesicles by removal of the ribosomes (92). These procedures, however, inherently alter in some way the biochemical characters of the components of interest. The other approach, to separate subcellular components by noncentrifugal methods, has probably not yet received the attention it deserves, and a more vigorous search should be made for alternative physical separation techniques compatible with preservation of the integrity of subcellular structures and functions. Examples that have already led to successful applications are phase partition (101), free-flow electrophoresis (102), differential filtration through membranes of graded pore size (103, 104), gel filtration (55), and immunoadsorption (93). With these and other tools still to be developed, tissue fractionation will continue for a long time to provide an essential bridge between morphology and biochemistry, as it has done so successfully in the past.

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