ANALYTICAL CYTOLOGY

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Methods for Studying Cellular Form and Function

Edited by

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foreword by

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Foreword

It is a thrilling experience to be engaged in research in a great scientific field at a time when that field is just coming to fruition, when new advances seem to be coming along with ever increasing frequency. Such is the situation in that synthesis of cell morphology and biochemistry which has come to be known as analytical cytology and which is ably dealt with in this volume.

A consideration of the time scale of these developments, contemporary to some of our elder colleagues, demonstrates the dynamic nature of the field.

Still less than half a century since the fundamental discoveries of von Laue and Debye in x-ray diffraction, and only three decades since Herzog began applying diffraction techniques to an analysis of biological structures, we have seen great advances not only in the determination of the macromolecular structure of tissue components but in the elucidation of the detailed intramolecular structure of proteins, carbohydrates, nucleic acids, and other complex substances.

Although electron microscopy is scarcely two decades old, the discoveries which have already been made in biomorphology suffice to indicate that cytology will soon have to be rewritten. The very morphological criteria by which we have now come to recognize cell organelles, such as mitochondria and other cytoplasmic particulates, would have been completely unfamiliar to the expert cytologist of a generation ago. We now identify fibrous proteins by their specific band patterns. Lipid-protein films only a few molecules thick, whose existence has long been postulated by general physiologists, have become old friends to the electron microscopist in the form of double contoured lines seen in thin sections of osmic-fixed tissue.

Highly sensitive optical techniques, applicable to *living* cells, have become available to the analytical cytologist. Polarization optics has been applied biologically for over a century, but it has been chiefly during the last thirty odd years (especially through application of the Wiener Theory) that advances have been most significant. Phase contrast and interference microscopy, relative newcomers on the scene, have already proven themselves indispensable to the experimental cytologist because of their ability to deal with very small

objects in the highly aqueous milieu of protoplasm. Ultraviolet, visible, and infrared microabsorption spectrophotometry, one of the most familiar tools of contemporary biophysics, has laid some of the very foundation stones in the edifice of analytical cytology thanks to its ability to determine the composition of microscopically resolvable objects in cells.

Cytochemistry, one of the infants in the family of analytical cytology, is nevertheless prolific. Perhaps the main reason for the greater significance of its contributions, despite the sometimes underestimated difficulties of exact localization and specificity of reactions, is that when the intracellular location of particular enzymes has been determined one has thereby learned much about the biochemical and physiological events which transpire at these loci.

Finally, the isolation of cell particulates and the determination of their composition and chemical reactivity has proved a milestone in the progress of analytical cytology not only in revealing the morphological sites of energy turnover and of biosynthesis in the protoplasmic microcosm but also in focusing the enthusiastic interest of biochemists and physiologists on cytological problems. What orthodox biochemists and physiologists of a generation ago would have confessed to an interest in mitochondria? These were the fixation artefacts which plagued their cytological colleagues and were therefore beyond the pale of respectable biochemistry and physiology! We dare not play too loudly on this theme for now the fixation artefacts are with us in far more realistic form and, indeed, the mechanism of fixation has become a central problem rather than one to be dodged by one or another strategem.

All the various aspects of analytical cytology mentioned above and many others are discussed at length in the present volume. In many cases the treatment is sufficiently detailed to permit the reader to proceed directly to the laboratory without the necessity of studying other theoretical or practical treatises.

The determination of the detailed morphology of cellular components or the analysis of their chemical composition may seem to have limited biological significance, however much ingenuity, patience, and precision may be required to solve problems as complex as those dealt with in analytical cytology. Nonetheless, it may be assumed confidently that when such detailed information is in hand the next step, *i.e.*, the relation of physiological function to the interaction of the chemical groups in the various components of the protoplasmic systems, will follow naturally and spontaneously. Having progressed sufficiently in the analytical phase we, or more likely a future genera-

tion, may pass on to the next phase, *i.e.*, the discovery of the directive, regulatory, regenerative, and historical aspects which differentiate the organism from less complex systems. In the present phase of the evolution of cytology this volume may be expected to play a significant role.

Francis O. Schmitt

CAMBRIDGE, MASSACHUSETTS

Preface

The purpose of this work is to bring together in one volume some of the important contributions of physics and chemistry to the study and the analysis of cellular structure and function. This field of scientific investigation appropriately has been designated *analytical cytology* by Professor Francis Schmitt who describes its scope and dynamic nature in the foreword to this book.

The preparation of this volume was undertaken with the collaboration of a panel of authors who are identified with original work in the various fields under discussion. It is my immediate and foremost editorial obligation in this preface to thank these authors for their contributions.

This book reflects in part the recent resurgence of interest in the study of the cell. Notable in this respect are the formation of the International Society for Cell Biology in 1947 and the Histochemical Society in 1950; the appearance of new journals, *Experimental Cell Research* in 1950, the *Journal of Histochemistry and Cytochemistry* in 1953, *Acta Histochemica*, Jena, Germany, 1954, and the *Journal of Biophysical and Biochemical Cytology* in 1955, and a new review, the *International Review of Cytology* in 1952; and the publication of monographs by Frey-Wyssling, ("Sub-microscopic Morphology of Protoplasm and Its Derivatives," Elsevier Press, Inc., New York, 1948), Glick ("Techniques of Histo- and Cyto-chemistry," Interscience Publishers, Inc., New York, 1949), Caspersson ("Cell Growth and Cell Function," W. W. Norton and Co., New York, 1950), Lison ("Histochimie et cytochimie animale," Gauthier-Villars et Cie., Paris, 1952), Gomori ("Microscopic Histochemistry," University of Chicago Press, Chicago, 1952), Pearse ("Histochemistry," Little Brown and Co., Boston, 1953) and Danielli ("Cytochemistry," John Wiley and Sons, Inc., New York, 1953).

Emphasis in the present volume is placed upon physical analytical methods. Of the four great instruments of physical optics—the microscope, spectroscope, camera and telescope—analytical cytologists use the first three. That the microscope receives foremost emphasis is in keeping with the important historical and contemporary role played by this instrument in the biological and the medical sciences. As the

following pages disclose, the microscope has now been developed so as to utilize each of the fundamental attributes of a light wave, namely, the amplitude, the frequency, the phase and the plane of vibration. The forms of microscopy which thereby evolve and serve in the analysis of the cell include light, phase, interference, polarizing and fluorescence microscopy and visible, ultraviolet and infrared microspectroscopy.

As judged by the volume of the literature on the subject, chemical methods which yield tinctorial reactions that are analyzed with the microscope are among the most productive means of tissue analysis, and accordingly, these methods are discussed in this work.

Finally, the use of electrons (electron microscopy), x-rays (historadiography and x-ray diffraction) and radioactive isotopes in the study of tissues, cells, and their component structures is reviewed in this volume.

Other, equally worthy, analytical methods falling within the scope of this work have not been included because of the practical limitations of space. It is hoped that this deficiency will be met by the presentation of additional or alternative material in subsequent editions.

In content and style the volume is prepared to serve as a reference book for research workers, teachers, and graduate and advanced undergraduate students in the biological and the medical sciences. While the tissue cell is of foremost interest to investigators in the life sciences, it is hoped that physicists and chemists, aside from those already in the fields of biophysics and biochemistry, may find this book to be informative. In addition, individuals in industrial corporations dealing with scientific instrumentation for biology may benefit by its use.

My sincere thanks are expressed to Dr. F. O. Schmitt, who has kindly written the foreword; to Drs. C. P. Rhoads, F. W. Stewart and G. N. Papanicolaou, who have provided opportunities, facilities and counsel for the study of the tissue cell in health and disease; and to the Board of Trustees of the Sloan-Kettering Institute for Cancer Research, the American Cancer Society, the U. S. Public Health Service, the Damon Runyon Memorial Fund and the Albert and Mary Lasker Foundation, who have supported such work.

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Robert C. Mellors

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Methods in the Optical Spectral Region

CHAPTER 1 *Cytophotometric Analysis in the Visible Spectrum*

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Introduction

Of all the techniques of analytical cytology with the compound microscope, perhaps quantitative absorption analysis in the visible spectrum is destined to become most widely used. First and foremost, this tool offers the possibility of expressing in objective values the differences in intensity of natural color or of the stains and tests which have been developed by histochemists and cytochemists for localization of substances in cells (Chap. 2). Visual comparisons are of very limited use in resolving any question where variations in both concentration and cell volume are involved, but where the chemical and physical conditions are favorable for absorption analysis, it is not a difficult procedure to obtain data from which the relative amounts of absorbing substance can be computed over a wide range of concentration. Swift (111), for example, was thus able to estimate desoxyribose nucleic acid in spherical nuclei, where the highest concentration was over twenty times that of the lowest. This possibility of objective measurement of intensity should make a microspectrophotometer as essential a piece of equipment in any histological, pathological, or cytological laboratory as the spectrophotometer is in a modern biochemical laboratory.

Second, and of nearly equal importance, is the role of visible microspectrophotometry as an adjunct to biochemical and physiological studies which seek to localize chemical components and functions within the cell. Very often such studies involve an estimate of the number of cells in the analytical sample and a computation therefrom of the amount of the components in the average cell. This is an unjustifiable procedure and of dubious value unless it is supported by cytological studies of the variation within the group. For example, it was thus computed by biochemists that the "average nucleus" from a number of different tissues of an animal contained the same amount of desoxy-

ribose nucleic acid (DNA). This was interpreted as meaning that a constant amount of DNA characterized each nucleus—a quite unjustifiable conclusion, as shown by an analysis of a sample which included some billions of nuclei, of which, so far as these data show, a considerable proportion might have contained little or no DNA. This picture has been greatly clarified by microspectrophotometric studies of individual nuclei, which show that the range of DNA content is indeed small (perhaps no more than 10 to 15 per cent), in marked contrast to the protein content which can vary by one or two orders of magnitude. The major part of this chapter will be devoted to describing apparatus and techniques for this relatively simple type of analytical cytology.

Finally, beyond this application of immediate practical value, absorption analysis with the microscope has other potentialities which may ultimately prove to be of much greater fundamental importance. The major chemical components of cellular substance are probably, as a rule, bound up in complexes about which only a little is known from studying them as isolated biochemical fractions. It does not seem overoptimistic to hope that detailed absorption analysis of these cellular complexes *in situ* can shed much light on such problems as the degree of polymerization, the nature and extent of the linkages among proteins, nucleic acids, and phospholipids, or the number and types of free acidic or basic groups on a large molecule. Such studies appear to wait only upon vigorous and resourceful attack by teams of cytologists and physical chemists who are convinced of the urgency and priority of the problem. The more advanced instrumentation for this approach is discussed below.

Comparison of Cytophotometry with in Vitro Absorption Analysis

Since the development of the comparimeter by Duboscq nearly a century ago, absorption analysis has come into increasing use as a quantitative analytical technique. It is rapid; it can often be carried out with relatively impure samples; the sample can be very small; and the sample, if measured in visible light, usually remains quite unchanged by the irradiation and is thus available for other analytical techniques. Especially in biochemistry, these advantages often make absorption analysis the method of choice. There are many types of instruments: some depend upon visual matching, others contain electrical photosensory elements, and the most advanced types are combined with monochromators to make an instrument suitable for obtaining data for complete spectral analysis of a colored substance. With the most widely used instruments, the chemist can read on a dial a figure from which, by the simplest arithmetic, he can compute the concentra-

tion in the sample from comparison with a known concentration. Much of the facility with which such an analysis proceeds can be explained by the standardization. The absorption path is fixed in length by use of a standard absorption vessel, or cuvette; the validity of the concentration-absorption law of Beer is assured by measurement of a series of solutions of known concentration; and often the instrument has a scale which converts the data on relative light intensities into a reading of optical density, or extinction, of the sample solution from which by simple proportion the concentration can be computed. It is literally true that for many chemical analyses by light absorption one need have no knowledge of the absorption laws, which are expressed by the formula:

$$E = kcd$$

where E = the extinction ($\log_{10}$ of the reciprocal of the transmittance)
k = a specific constant
c = the concentration of colored substance
d = the thickness of the absorbing layer

Quantitative absorption analysis of a portion of a cell, however, is necessarily largely empirical, because it must cast loose from these mooring standards. First, the absorption path is determined mainly by the geometry of the cells; only in small part is it under control of the cytologist through adjustment of section thickness. Second, it is doubtful if for intracellular substances there can be any adequate standard comparable with the known solutions of the analytical chemist. Within the dimensions of most single cells the absorption path will be not more than a few microns, and consequently the concentration necessary to give detectable color must be approximately a thousand times that necessary for detection or estimation in an absorption cuvette, which is 1 cm thick. Nearly always this higher concentration within the cell is far above the range in which the substance can exist in the physical state prevailing in the lower range in the liquid system within the absorption cuvette. At the very least, within the cell it must be expected that a significant fraction of the total mass of any biological substance must be in aggregations considerably above that of homogeneous unimolecular dispersion which predominates in the dilute liquid. At still higher concentrations, for example, those of proteins [40 to 60 per cent in nucleoli (116)] or nucleic acids [DNA, 23 per cent in normoblast nuclei (88)] the physical state must be close to that of a solid system. The extinction coefficient (k in the formula above) is markedly dependent upon these physical states, and it seems highly questionable whether any artificial concentrated system, such as a gel or a precipitated fiber, is likely to resemble the physical state within the cell

closely enough to justify the obvious procedure of using it as a cytological standard in a cuvette of cellular dimensions.

The high concentration is but a part of the difficulty. Within the cell perhaps very few, if any, biological substances with reactive groups exist in high concentration as free ions or molecules; instead, the proteins, nucleic acids, acid polysaccharides, phospholipids, etc., which are the principle chemical constituents of protoplasm appear to be bound together in complexes of complexity as yet indefinable. Certainly, therefore, there is no adequate basis for any prediction as to the deviation within such systems from the extinction coefficient, which is characteristic of one particular chemical component when it is in a dilute liquid system. The deviations in complexes may quite possibly be comparable in magnitude to those due to aggregation and change from liquid to semisolid state. Furthermore, it must not be overlooked that the cell analyses of the sort to be described below are generally carried out upon tissue which has been fixed, a process which profoundly alters both aggregation states and the heterogeneous system—so that any standard would presumably be valid only for a *particular* molecular complex with one type of fixation. For example, a widely used type of nucleoprotein fixation is that in acetic alcohol, which must remove a large part of the lipid from the complexes, while there are other fixations which will hold all three of these major constituents in the cell throughout long technical procedures (p. 1/8 and Fig. 1·2).

The considerations just enumerated apply to studies of natural absorption, such as that of hemoglobin, chlorophyll, and plant pigments in the visible spectrum, and that of proteins, nucleic acids, ascorbic acid, etc., in the near ultraviolet range. But the most widely used methods of cytological localization and photometric analysis involve artificial color developed as a specific test or as a specific addition of a dye, and, therefore, there is the further difficulty, perhaps the single greatest chemical complication, that the aggregation and complex formation undoubtedly have an effect upon any quantitative interpretations from data obtained in photometric measurements on parts of a cell.

These are but some of the chemical difficulties which beset the *cytophotometrist*, as Moses (72) has called the student of absorption analysis of the cell; there are others of a chemical nature and, in addition, many physical difficulties (p. 1/31). One would be tempted to stress only these aspects and to discourage all attempts to explore the possibilities of cytophotometry [see, for example, Glick (36)] if he were not convinced of the undoubted urgency and priority of the problem of getting quantitative chemical information at the level of the individual cell. To a general cytologist, a cell-minded biochemist, or an alert cellular physiologist, there can be no question but that this is one of the essential

first steps in progress toward the understanding of the fundamental intracellular chemistry, or chemistry of protoplasm, which Claude Bernard so clearly visualized as the shining goal of general physiology. The cytologist knows that, except for the possibility of electron microscopy, the attempt to find striking evidences of the correlation of cellular morphology with functional phenomena long ago reached a dead end (101). The biochemist is acutely aware that his pursuit of complex, vital chemical cycles led him to postulate the existence of the very sort of chemically heterogeneous, organized entities that the cytologist has been studying as elements of cellular morphology (107, 38). The general physiologist must be impressed by the fact that, except for special aggregates of inert stored material, the active protoplasm of cells is remarkably the same in containing high concentrations of proteins, nucleic acids, and phospholipids—and that if one is to study fundamental aspects of growth, normal or abnormal, he must concern himself with the intracellular changes in these main chemical constituents of protoplasm (27). Especially during the past decade many individuals in these groups of biologists have reoriented their research toward the chemical aspects of the cell.

Since the cytophotometrist's sample is so very different from the dilute solution of the chemist, it is obvious that the attempt to develop quantitative techniques must be deeply concerned with the problem of how the absorption laws operate within the cellular substance. There are no data on these intracellular physicochemical systems from which this can be predicted, and hence there must be a large element of empiricism in the development of cytophotometry. Wherever possible, absorption data on cells must be checked for conformity to the Beer-Lambert relationship by reference to data from direct chemical analysis or by arranging the experimental details so as to give an intrinsic check on the validity of the absorption laws. This course has been followed in the development of visible microspectrophotometry, but it was almost wholly ignored during the early years of application of ultraviolet absorption analysis to cells (16). For example, it is pertinent in the case of much cytological material to question whether Lambert's law ($E = kd$) holds, since (*a*) the test or stain may be developed differently on the surfaces and in the interiors of the section, or (*b*) there may be orientation of absorbing groups so that they are not random with respect to the wavefront of the light (p. 1/47). That these do not operate to cause major deviations from the Lambert law was demonstrated by showing conformity over a wide range of section thickness (94). Far more serious is the possibility that Beer's law ($E = kc$) may not hold over a concentration range encountered in a series of cells. There are many examples where, if one assumes conformity with Lambert's law,

adherence to Beer's law has been checked by comparison with direct analysis. Thus, the cytological determination by the Feulgen reaction of relative DNA content of individual cells in a series of related fishes was found to conform closely to the results obtained by direct analysis of a mass of cells (98); while the cytophotometric evidence of protein loss from nuclei treated with one isolation technique was confirmed by later results of direct analysis of isolated nuclei (p. 1/65). Flax and Himes (30) have made a particularly illuminating study of the validity of Beer's law for the basophilia (azure B) of ribonucleic acid in tissues. Nonconformity is most readily detected as alteration of curve shape. These authors found that for a variety of animal tissues the shape of the absorption curve did not change, and they therefore concluded that photometric measurements are valid to determine the relative amounts of dye bound to substrate in different cells. This did not, of course, resolve the question of the extent to which the bound dye measures the relative amounts of nucleic acid present in the tissue. But, in separate experiments on the cytoplasm of mouse oöcytes, Flax (28) has found that the same relative values were obtained from cytophotometric measurements of the basophilia by visible light and of the natural absorption of the nucleic acid in ultraviolet light. A similar correlation has been shown for the Feulgen reaction for DNA in nuclei (117).

Comparison of Cytophotometry with Cytology

The preparation of cytological material for photometric chemical analysis has been found to have rather different requirements from those developed primarily for cellular morphology. For morphological cytology, fixation is selected to preserve cells with a lifelike appearance (Fig. 1·1*A*), or to facilitate the application of stains or metallic impregnation which will give the maximum contrast among the component parts of the cell or tissue; for photometric analysis, these considerations are often secondary to others, such as the following: (*a*) that the fixing fluid produce no color in the tissue, (*b*) that the tissue be left in such a condition that chemical staining or tests can be carried out, (*c*) that the tissue be in such a condition that chemical extractions or digestions can be carried out, and (*d*) that refractive index differences among cellular or tissue elements be minimized. Fulfilling these requirements has involved considerable sacrifice of cellular morphology in the technique which has been most widely used for nucleoprotein, namely that of fixation in Carnoy's acetic alcohol mixture (Fig. 1·1*B*); the mitochondria are destroyed, the chromatin is clumped instead of being fairly evenly dispersed throughout the nucleus, and much material is lost from the zymogen granules. The most successful coprommise be-