BIOCHEMICAL CYTOLOGY

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PREFACE

We have attempted in this book to present an integrated version of the facts which today are known about the morphology and the biochemistry of the cell. There exist many excellent textbooks of cytology and biochemistry. What remains to be done is the difficult and important task of linking these two sciences more closely together, now that they have so much in common. This is what we have tried to do, with the hope that the book will prove useful to many advanced students and research workers.

It has been assumed that the reader already knows the fundamentals of descriptive cytology, biochemistry, embryology and genetics. Our goal will have been reached if he enjoys the attempt we have made to show that structure and metabolism are so closely linked together that they cannot be separated.

Special emphasis has been laid on the problems which are most familiar to the author. This will perhaps excuse the apparent unbalance of the book: if too much is said on embryos in Chapter VIII and too little on cancer cells in Chapter IX, it is because the author has spent a good deal of his life working with embryos and has so far never touched a cancer cell.

The emphasis has been laid on the more dynamic aspects of cytology, rather than on detailed description: more is said about nucleocytoplasmic interactions in unicellular organisms and embryos than about the pure description of cytoplasmic and nuclear constituents.

Hypotheses and personal opinions have not been forgotten, for hypotheses, provided they can be experimentally tested, may become more important than dry facts. Ideas are as vital to scientists as engines to cars or airplanes. Nowadays, some scientists forget that thinking may sometimes be more useful than performing an experiment.

The book has been written directly in English and the author may not have expressed the ideas and facts as precisely as he would have wished. But what has been lost in subtlety has perhaps been gained in directness and clarity.

While this book is a mere expansion of the lectures given by the author to his students in the University of Brussels, its origin is, however, different. It is the direct result of a series of lectures given at the Indian Cancer Research Centre in Bombay, in January and February 1956.

It was in Bombay that the first chapters of the book were written. Thanks to our Indian hosts, especially Dr. V. R. Khanolkar and Dr. A. R. Gopal Ayengar, we found continuous encouragement and ideal conditions for writing. The discussions we had with many of the members of the Cancer Research Centre in Bombay greatly contributed to the writing of the book. Our sincerest thanks go to all of our Indian colleagues for their continuous kindness and interest.

Several of our colleagues and co-workers kindly agreed to read the manuscript and to improve the English. Doctors A. R. Gopal Ayengar, H. Peters, W. A. Jensen, R. Logan and, especially, P. Couillard deserve all our thanks for their kind help; important remarks and suggestions were made by them. Other criticisms came from Dr. F. Haguenau (Paris), who read Chapter III, and from a number of friends and colleagues from the University of Brussels; without the help of Professors R. Jeener and H. Chantrenne, Doctors R. Thomas and A. Ficq, many errors would have escaped notice.

Considerable help, for which it is impossible to give adequate thanks, came from all those who contributed to the preparation of the illustrations: many beautiful photographs were kindly sent to us by Dr. W. Bernhard, Dr. R. Briggs, Prof. H. Callan, Dr. H. Fraenkel-Conrat, Dr. H. Gay, Dr. A. R. Gopal Ayengar, Dr. F. Haguenau, Prof. D. Mazia, Dr. Y. Moulé, Dr. K. Porter, Prof. H. Ris, Prof. F. Sjöstrand and Dr. W. Vincent. The original photographs were made, in our laboratory, by Mme. E. De Saedeleer, Doctors A. Ficq, M. Steinert and F. Vanderhaeghe, while Professor H. De Saedeleer was so kind as to redraw and improve a number of the text figures. The long and unpleasant job of typing the manuscript, reading the proofs, and preparing the subject index has been performed by Mme. E. De Saedeleer; it is impossible to say just how patient and efficient she has been.

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CHAPTER I

INTRODUCTION: THE RECENT HISTORY OF BIOCHEMICAL CYTOLOGY

A short survey of the advances made in biochemical cytology over the last thirty years immediately shows that the present book, which deals mostly with problems of current interest, will very soon be outmoded.

When the author, then an undergraduate medical student, first started working in a laboratory in the late twenties, he was asked to choose between two problems: a study of the production and role of mitogenetic rays in developing embryos, or an investigation of the localization of thymonucleic acid in growing oöcytes, with the aid of the newly discovered Feulgen reaction. Fortunately, he preferred chemistry to physics and decided on the second problem—mitogenetic rays are now completely extinct and there would be no profit in trying to dig them out of the grave! Very little was known, at the time, about nucleic acids; "thymonucleic" acid was supposed to exist in animal cells only and to contain in its molecule some queer derivative of glucose; it was identified by Levene and Mori (1929), a little later, as deoxyribose. "Zymonucleic" acid, containing a pentose, was thought to be a specific plant constituent, but it was known that pancreas, for some mysterious reason, was rich in this "plant nucleic acid." These two substances have had since those days a very distinguished career indeed; thymonucleic acid is now known as deoxyribonucleic acid (DNA); it is present in the nuclei of all cells and is thought to be important in the transmission of hereditary characters; ribonucleic acid (RNA) is also present in all cells, animal or vegetal, and is believed to play a significant part in protein synthesis.

So little of this was known in 1930 that the only function one could suggest for DNA was the role of a pH buffer inside the nucleus. The discovery of the nuclear localization of DNA, however, marked distinct progress, in part because it was made by the utilization of one of the first really specific and elegant cytochemical reactions, the Feulgen (Feulgen and Rossenbeck, 1924) reaction. It gave the hope that the location in the cell of many substances of biological interest might be discovered. In this respect, Lison's book ("L'histochimie animale") markedly contributed, in 1936, towards the emergence of cytochemistry as a new discipline. Unfortunately, most cytologists had, in those days, very little

interest in genetics or biochemistry. The recognition of the genetic role of DNA was to come some ten years later and from a rather unexpected field, microbiology (Avery *et al.*, 1944; Boivin *et al.*, 1948). But, in 1930, bacteria had no genes, not even nuclei!

The story of RNA, the old "plant nucleic acid" is no less interesting. Its existence in animal cells other than pancreas was established in studies on sea urchin eggs. J. and D. Needham (1930) had found that the nucleic acid phosphorus content of the egg does not increase from fertilization up to the pluteus stage despite the tremendous multiplication of the nuclei. They drew the conclusion that the eggs contain a DNA reserve in the cytoplasm; this reserve is utilized for the building up of innumerable nuclei. Such a conclusion, however, cannot be accepted, since the cytoplasm of the unfertilized eggs is Feulgen-negative, and chemical analysis shows only traces of DNA in it. On the contrary, the plutei stain intensely with Feulgen and contain large amounts of DNA. A marked synthesis of DNA thus occurs during development. If the total nucleic acid is really constant, one can hardly escape the following conclusion: the cytoplasm of the unfertilized egg contains large amounts of RNA which, during development, is in part converted into DNA (Brachet, 1933). This "conversion" hypothesis has now been abandoned since the methods used at the time lacked the necessary specificity; and the total nucleic acid content, in fact, increases during development. But it remains that DNA is being synthesized when the nuclei multiply and that sea urchin eggs contain a large store of RNA. Most of it is necessarily localized in the cytoplasm, because of the very small size of the nucleus in unfertilized sea urchin eggs (female pronucleus). Ribonucleic acid is therefore no more a "plant" nucleic acid than DNA is an "animal" nucleic acid.

Where exactly is the RNA localized inside the cell? Patient efforts to solve that problem have long been frustrated because textbooks said that methyl green is an excellent stain for nucleic acids. However, cells rich in RNA and poor in DNA (unfertilized sea urchin eggs or yeast cells, for instance) completely fail to stain with this dye. Methyl green is, therefore, a good stain for DNA, but not for RNA. When one uses a mixture of two basic dyes, methyl green and pyronine, which Unna advocated many years ago and which was used very little around 1935, one finds that the RNA-rich cells stain intensely in red with pyronine. More time and work were necessary before it could be proven unequivocally that the material stained by pyronine actually is RNA. The proof came when sections of the RNA-containing cells were treated with ribonuclease, an enzyme which digests RNA in a specific way. When the sections, which had undergone previous ribonuclease digestion, were stained with Unna's

mixture, they no longer took pyronine. The principle of the method is so simple that one might wonder why the author (Brachet; 1940) did not think of it much earlier: the reason again is that many textbooks advised against the use of ribonuclease as a cytochemical tool (it had been used by Van Herwerden, as early as 1913) because of its alleged lack of specificity. It is fair to say, however, that the enzyme was strongly purified in 1938 only (Dubos and Thompson, 1938) and crystallized in 1940 (Kunitz, 1940).

When the distribution of RNA was studied in various tissues by Caspersson (1941) and the author (1941) by two different techniques, the same general conclusion emerged: RNA is localized in the nucleoli and the cytoplasm of the cells, and a cell's content in RNA is directly linked to its protein synthesizing capacity. The view that RNA plays a role in protein synthesis has been easily accepted by many cytologists, but most biochemists have long been reluctant to agree with it. It is curious that one had to wait until very recently (1954) before Gale and Folkes proved that digestion of RNA with ribonuclease inhibits protein synthesis: ribonuclease was being used by cytochemists for fifteen years before straight biochemists had the excellent idea to utilize it in biochemical systems also.

It has been shown very recently that the enzyme ribonuclease can actually penetrate inside certain living cells and stop protein synthesis *in vivo*. Here again, those textbooks which state in a dogmatic way that proteins are unable to cross the cell membrane barrier have not only been wrong, but harmful, because they have hampered scientific progress.

Similar stories might be told about many other aspects of cell physiology. For instance, the problem of the role and chemical composition of the various cell constituents is obviously one of the utmost importance, yet, it has been ignored for many years. We have known, since Warburg's and Keilin's days, that small granules, important for cellular oxidations, leak out of disrupted cells; but it took many years before Claude, who had the training of a cytologist, found that, by careful centrifugations of homogenized cells, it is possible to separate, in relatively pure form, nuclei, mitochondria, and microsomes. In this case, as in so many others, no real progress was made until Claude (1943) had the curiosity to look carefully at the homogenate under the microscope.

A few conclusions can be drawn from this brief and incomplete outline of the history of cell physiology in recent years. First of all, overspecialization is to be carefully avoided. The biologist who is interested in cell physiology should not be a morphologist, or a physiologist, or a biochemist: he should not only be capable of using physiological and biochemical methods as well as the microscope, but he should utilize them all in attacking his problem. Neither the variety of the methods nor the acquisi-

tion of a wide knowledge in very different fields should frighten him. This is the price which has to be paid if cell physiology is to progress. The same price has been paid in the field of biochemical genetics of microorganisms, in which such outstanding advances have been made recently.

The basic knowledge necessary to undertake research in cell physiology will be found in textbooks about cytology, biochemistry, and genetics; many of them are extremely valuable, but none of them should be taken as God's own word. Facts are more important than statements made in textbooks by eminent authorities.

The present book is to be regarded with more caution still, since it deals with recent rather than well-established facts and ideas: many of the facts and ideas presented here will prove in the near future to be partially or entirely wrong. However, the book will have served its purpose if one of its readers is interested enough to design and perform the experiments needed in order to show what is wrong and what is right.

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Chapter II

BRIEF SURVEY OF THE TECHNIQUES

As has already been pointed out in the Introduction, the study of cell physiology calls for a very large number of entirely different methods. It would be fruitless to present them here in detail since these techniques continuously undergo improvements and modifications. We will thus limit ourselves to a brief survey of the methods most commonly used in cell research, and deal successively with optical methods, cytochemical techniques, quantitative histochemistry, and the isolation of cell constituents.

I. OPTICAL METHODS

The classical technique of ordinary microscopy remains of course the basis of any cell study, whether the observations are made on living cells (sometimes vitally stained with neutral red or Janus green) or on sections (or smears) of fixed and stained tissues.

Phase contrast microscopy and *interference microscopy* (see the recent reviews of Osterberg, 1955 and Barer, 1956) constitute a very useful complement of light microscopy; phase contrast equipment often shows many details which cannot be seen with the ordinary microscope, and it has been very successfully combined with microcinematography for the study of cells cultivated *in vitro* (Fig. 1). Interference microscopy, however, is likely to become much more important than phase microscopy for the cytochemist, because it enables one to measure the *dry mass* of parts of the cell (Barer, 1953, 1956; Davies *et al.*, 1953); for instance, Davies *et al.* (1954b) have shown with this method that, in amoebae, the contractile vacuole is very poor in dry substance while the food vacuoles are richest. The technique, which is not too elaborate, compares favorably with the much more complicated X-rays spectrography method, as shown by Davies *et al.* (1953). The main interest of the interference microscope is that it provides a very valuable reference unit for other cytochemical measurements; the apparatus also makes it possible to follow the loss in dry substance of a definite cell constituent (the nucleus, for instance) when the section or the smear is treated with a given extraction medium; for instance, it has been possible to measure in this way the nucleoprotein content (80–85%) of the chromosomes (Mellors, 1955) and to estimate the loss in substance which occurs when nuclei isolated in nonaqueous

media are treated with solutions of citric acid or sucrose (Hale and Kay, 1955).

Of great importance for the cytochemistry of nucleic acids is, since Caspersson's pioneer work in 1936 and 1940, the *ultraviolet microscope*. The instrument, a schematic early version of which is represented in

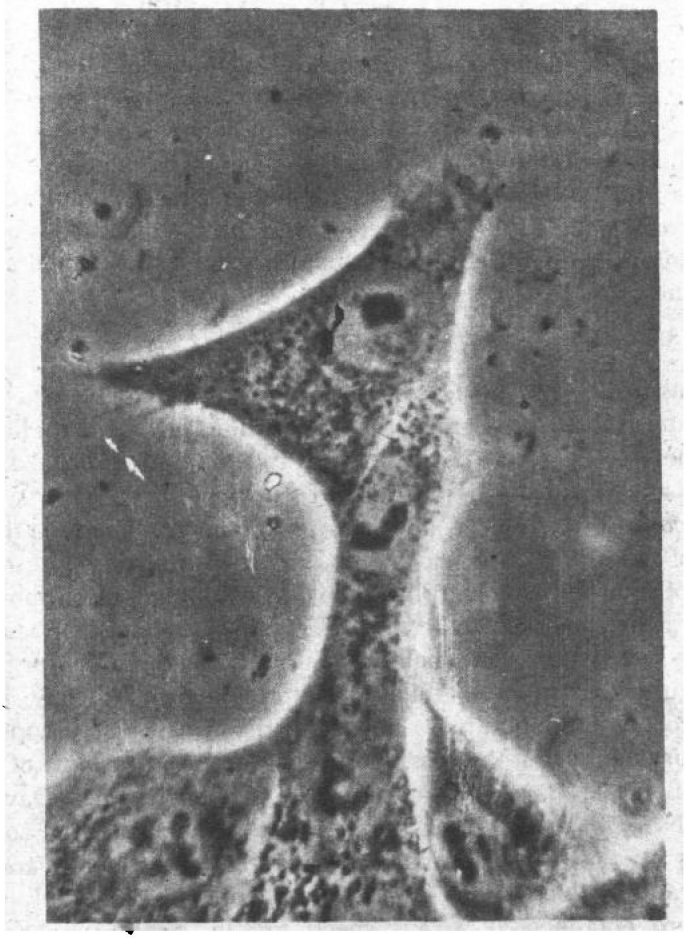


FIG. 1. Phase contrast microphotography of a living cell. (Original photograph by Dr. M. Steinert.)

Fig. 2, has been progressively improved by Caspersson and his co-workers (see Caspersson and Walker, 1955–1956 for recent types of refined apparatus). The greatest interest of the technique is that it enables one, with the help of a quartz microscope, a monochromatic source of ultraviolet (UV) light, and a photomultiplier attachment, to measure the complete UV absorption spectrum of a very small part of the cell. Since nucleic

acids have a very high UV absorption around 2600 Å, due to the presence of purine and pyrimidine bases in their molecule, it is possible to detect and, in principle, to evaluate the nucleic acids of small cellular constituents. The development of scanning techniques (Caspersson *et al.*, 1951) now permits the estimation and automatic registration of the nucleic acid content of the whole nucleus. It should be kept in mind, however, that many sources of errors, largely due to the heterogeneous nature of the biological material, exist and, if possible, should be corrected. For

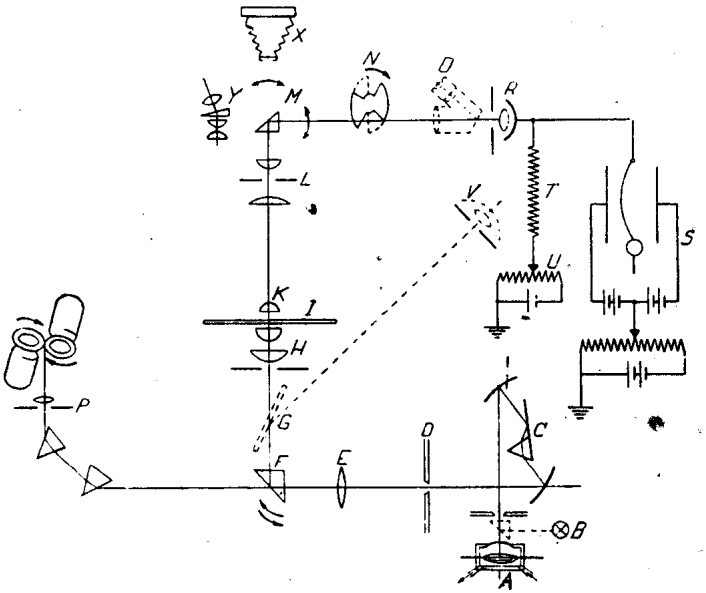


FIG. 2. Ultraviolet microscope of Caspersson. *A, B,* and *P:* light sources, *C:* monochromator, *H, K, L:* quartz microscope, *F* and *M:* quartz prisms, *N:* rotating sector, *O:* telescope, *R:* photocell, *U:* potentiometer, *S:* electrometer.

instance, fixation considerably modifies the UV absorption of the various parts of the cell (Davies, 1954).

A similar type of apparatus, but with a reflecting microscope instead of a quartz microscope, has been devised by the group of Wilkins and Davies and others, at King's College, London. Among other things, Deeley *et al.* (1954) and Walker and Deeley (1956) have recently described scanning methods, and Davies *et al.* (1954a) a simple technique for crushing cells prior to the measurements.

While there is no question about the usefulness of the UV microspectrophotometric methods, it seems that the technique (which is complicated and extremely costly) perhaps yielded its most important results in Caspersson's hands more than ten years ago, when he was able to trace

the localization of RNA in many different types of cells. But the more recent technical improvements developed in Caspersson's and Wilkins' laboratories have none the less their importance for future work, which will now become more and more accurate and quantitative.

The situation is somewhat different with *electron microscopy*, which still is in its beginnings: not only are the electron microscopes themselves being steadily perfected, but the techniques for fixation and cutting ultrathin sections have been immensely improved (reviews by T. Anderson, 1956 and Sjöstrand, 1956). Many cytological details have been discovered since Porter *et al.* (1945) published their first electron micrographs of cells which "really looked like cells." As we will see in the next chapter, beautiful photographs of mitochondria and other cell organelles have now been obtained in many different laboratories. A few warnings should, however, be issued concerning the interpretation of the results obtained with present methods of electron microscopy. The most important is that we do not know exactly the nature and magnitude of the artifacts resulting from fixation, sectioning, drying, and submitting the specimen to the flux of electrons. The higher the magnification, the more likely are these artifacts to appear. Another reason for some concern is that almost all the "nice" electron microscope photographs so far published have been obtained after buffered osmium tetroxide fixation: it is to be hoped that, as already done by Lehmann (1954) and by Bretschneider (1954)—who are very well aware of the pitfalls of electron microscopy—a wider range of fixatives will be used. The need for more eclecticism in this choice of fixatives becomes obvious when it is realized that, after buffered osmium tetroxide fixation, the cell organelles which have reduced the fixative show up much better than the rest of the cell; osmium tetroxide is easily reduced by certain lipids and it may well be that the cells contain many important inclusions which, if they are devoid of these lipids, escape detection with the electron microscope.

Although it is not exactly an optical method, a few words should be said about *microdissection*. The required instruments have been considerably improved in recent years, so that the introduction of microneedles or micropipettes into the cell is now a relatively easy matter. Details about the various types of micromanipulators and the experimental possibilities they afford can be found in a recent and well-documented review by Kopac (1955).

II. CYTOCHEMICAL METHODS

The essential principle of all cytochemical methods is to apply a chemical test, specific for the detection of a certain chemical substance, to histological sections: the localization of the reaction product is then

observed under the microscope. As pointed out by Lison, as early as 1936, there are two absolute prerequisites if any cytochemical reaction is to be of value: the reaction must be highly specific and the localization of the reaction product (which is usually colored) must remain unchanged during all manipulations. Nonspecificity, and diffusion of substrate or reaction products remain the two pitfalls in cytochemistry.

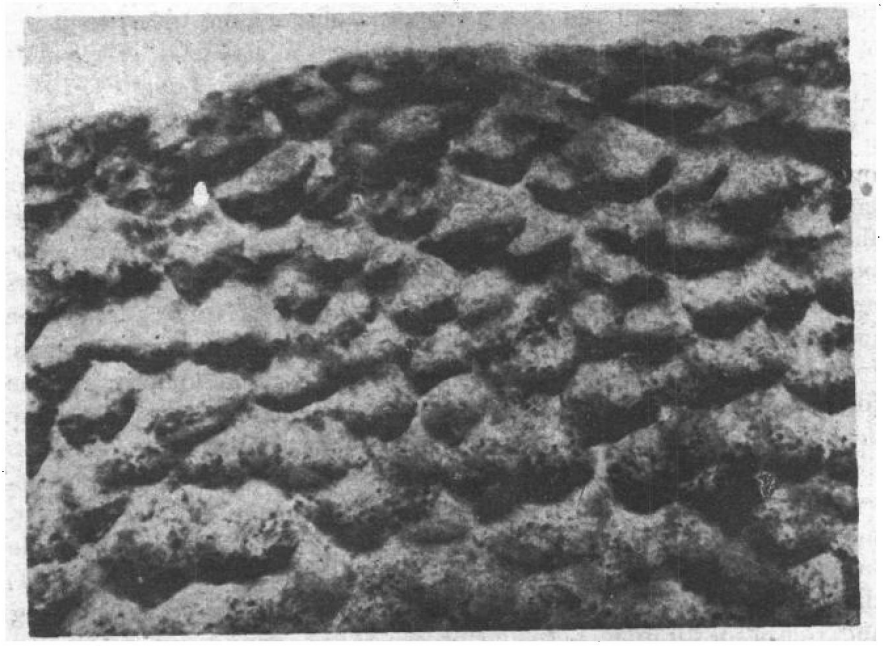
A very large number of cytochemical methods exist nowadays; they have been described and adequately discussed in recent books by Lison (1953b), Pearse (1953), and Glick (1949). The latter also published, in 1953, an interesting review article on the many difficulties which may occur in the interpretation of cytochemical observations. Because of this wealth of information, it will be sufficient here to give a list of the more valuable cytochemical techniques and to criticize them briefly.

The problem of *fixation* has been ably discussed by Wolman (1955) in a recent review. Several workers have been studying the effects of the ordinary histological treatments (fixation by chemical agents, dehydration, embedding, etc.) on the chemical composition of the cell; for instance, the nucleic acid and protein content and the phosphatase activity (Norberg, 1942; Sandritter and Hartleib, 1955; Hartleib *et al.*, 1956; Harbers and Neumann, 1955; Michel *et al.*, 1956; Davies, 1954, etc.) have been measured quantitatively in fixed and embedded tissues. In many instances, freeze-drying[1] (i.e., fixation at very low temperature, in liquid isopentane for instance, and dehydration *in vacuo* at low temperature before the final paraffin embedding) or freeze-substitution (in which the tissue is fixed in the same way and then treated with cold methanol in order to dissolve the ice crystals) have obvious advantages: diffusion of substances such as glycogen is avoided during fixation (Fig. 3) and hydrosoluble substances are not extracted (see the recent review of Bell, 1956). It should, however, be borne in mind that some enzymes resist freeze-drying and freeze-substitution; autolytic processes may thus occur as soon as the section is placed in contact with water: extensive autodigestion of proteins and RNA, by the pre-existing proteolytic and nucleolytic enzymes, has often been observed, in our own laboratory, when pancreas or intestine had been fixed by freeze-drying or freeze-substitution.

The cytochemical detection of lipids is still imperfect: dyes (Sudan III, Sudan black, etc.) which are soluble in lipids, but not in water, are useful for the detection of fatty reserve droplets. A special type of phosphatide, plasmalogen (or acetalphosphatide), can be easily detected

[1] The principle of freeze-drying was perhaps imagined by the French writer Edmond About in his famous novel "L'homme à l'oreille cassee." A colonel of the Napoleonic great Army was frozen in Russia and then dehydrated *in vacuo;* he eventually revived, after being rehydrated.

since it gives the well-known Schiff aldehyde reaction (violet color with fuchsin-sulfurous acid) after denaturation of the proteins: this is the plasmal reaction of Feulgen and Voit (1924). It is worth pointing out that the Sudan stainable droplets and the plasmalogen-containing granules have an entirely different localization inside the cell: the former collect at the centripetal pole with the fat droplets when the cell is strongly centrifuged *in vivo;* plasmalogen, on the contrary, is entirely bound to cell granules (mitochondria, microsomes) which migrate to the centrifugal pole.



(a)

FIG. 3. Glycogen detection in liver: PAS reaction. Chemical fixation (*a*), producing diffusion artifacts, and fixation by freeze-substitution (*b*).

Very good methods are in existence for the demonstration of glycogen and glycoproteins. They are also based on the Schiff aldehyde reaction, which becomes positive when the glycol groups of the hexoses are oxidized with periodic acid (periodic acid-Schiff (PAS) reaction of Hotchkiss, 1948 and McManus, 1948). The utilization of salivary amylase, for the specific digestion and removal of glycogen or starch from the cells, remains a useful additional test (Fig. 3). Furthermore, glycoproteins give a metachromatic (red) staining with toluidine blue (Lison, 1935) and