CYTOLOGICAL TECHNIQUE

JOHN R. BAKER

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The Principles and Practice of Methods used to determine the Structure of the Metazoan Cell

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DEDICATED WITH GRATITUDE
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FORMERLY
PROFESSOR OF ZOOLOGY
AND
COMPARATIVE ANATOMY IN THE
UNIVERSITY OF OXFORD

'. . . in der Mikrotechnik nichts selbstverständlich ist. Im Gegenteil gilt hier der Satz: Es ist selbstverständlich, also muss es eigens gesagt werden.'

S. V. APÁTHY (1912)

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PREFACE TO THE SECOND EDITION

MORE than a decade has passed since the first edition of this book was published. This new edition follows the same general plan as before, but nearly the whole of it has been re-written with a good deal of expansion and modernization. The account of the structure of cells given in the first chapter has been profoundly changed as a result of recent discoveries, and I hope it may prove useful to many who do not want to study the rest of the book in detail. Embedding has been much more fully treated than in the first edition and a section on celloidin added.

Although this book deals only with one kind of cytological technique, yet it bears on other kinds. It is concerned with structure, yet provides a basis for histochemical studies; it is cytological, but adapted to the needs of the histologist and pathologist; it deals with Metazoan cells, but can be used by the protozoologist and botanist. It contains a good deal of information that has not been published previously.

I am very much indebted to friends who have given valuable advice about this new edition, especially Dr. W. Holmes, Mr. P. B. Medawar and Mr. G. M. J. Schmidt, who have shown me errors in what I wrote before and have generously gone to a lot of trouble to answer questions on difficult points. (No error in this edition is attributable to them, for they have not seen the manuscript.) I thank Professor E. S. Goodrich once more. All

my work in cytology has been done in his Department and I owe much to his advice and encouragement.

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PREFACE TO THE FIRST EDITION

THIS book is founded on the experience gained in giving nine annual courses in cytological technique to advanced students in the Department of Zoology and Comparative Anatomy at Oxford. I want to express my gratitude to Professor E. S. Goodrich, F.R.S., in whose Department it has been my privilege to work since I was an undergraduate, and to Dr. H. M. Carleton, from whom I first began to learn cytology fourteen years ago. I cannot omit to mention my indebtedness to Mr. Frank Sherlock, whose manipulative skill in microtomy is well known to all Oxford zoologists.

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CHAPTER I

INTRODUCTION

CYTOLOGICAL technique is co-extensive with cytology, and the whole subject could not be covered even superficially in such a small book as this. I have confined myself to the techniques of making permanent preparations, especially sections, intended to show the structure of the cells of many-celled animals under the microscope. Wonderful advances are being made in various other branches of cytology. Perhaps the most striking is the actual isolation of mitochondria and other cell-constituents, a technique that we owe to the pioneer work of Bensley and Hoerr (1934). Old techniques introduced by Raspail in 1829 and Altmann in 1890 have been revived to give us micro-incineration and the freezing-drying method once more. Micro-manipulation enables us literally to probe the living cell. Histochemistry has made great advances: enzymes have actually been made to reveal their presence by their action in sections, and ultra-violet spectrophotometry has taught us much about the distribution of nucleoproteins in cells. The electron microscope makes us hope for still minuter knowledge of cellular structure, while X-rays are revealing details of the structure of proteins far beyond anything that the ordinary microscope can detect. We may hope that other advances, as wonderful as these, may be made in the not-far-distant future. Amid all the excitement of recent cytological research, it may seem a little prosaic to hark back and look at ordinary stained sections. Nevertheless, cytology probably owes more to the staining of sections than to any other technique. Anyone who doubts this should ask himself how much we should know about chromosomes without the aid of this method. Whatever kind of cytology interests us

particularly, we are likely to require a background of the kind of knowledge that only stained sections can give. Further, an understanding of the physics and chemistry involved in the making of stained sections can be helpful in almost every branch of cytology.

My guiding principle has been to give as few methods as possible, and to describe them in great detail, with full information as to the nature of the reagents used. It may be urged against this book that the techniques are not set out in such a way that one may refer to them quickly. That is true, for this is by no means a recipe-book. There are plenty of recipe-books and they serve a purpose, but it is not the purpose of this book. I have tried to explain the *principles* of the methods, and also to teach thoroughly the *practice* of them to those who want patiently to understand what they are doing at every step, so far as that is possible.

All irrational methods, with which the literature abounds, have been carefully avoided. Many investigators have published formulæ for fixatives and other fluids, without giving any indication of the researches that led up to them. They have given no concrete evidence of why they are to be preferred to other fluids, nor of whether various proportions of the substances used were tried, with what results. Often they have mixed oxidizers and reducers, or acids and basic substances. In such cases one does not know to what extent the results obtained are due to the original components, and to what extent to the results of their reactions. One is working in the dark, without knowing what one is doing. Such irrational methods often give satisfactory results, but the results cannot be properly interpreted and they are not better than can be obtained by rational methods. Such considerations have led me to omit many well-known and much-used techniques. It is to be hoped that cytological technique will become a truly

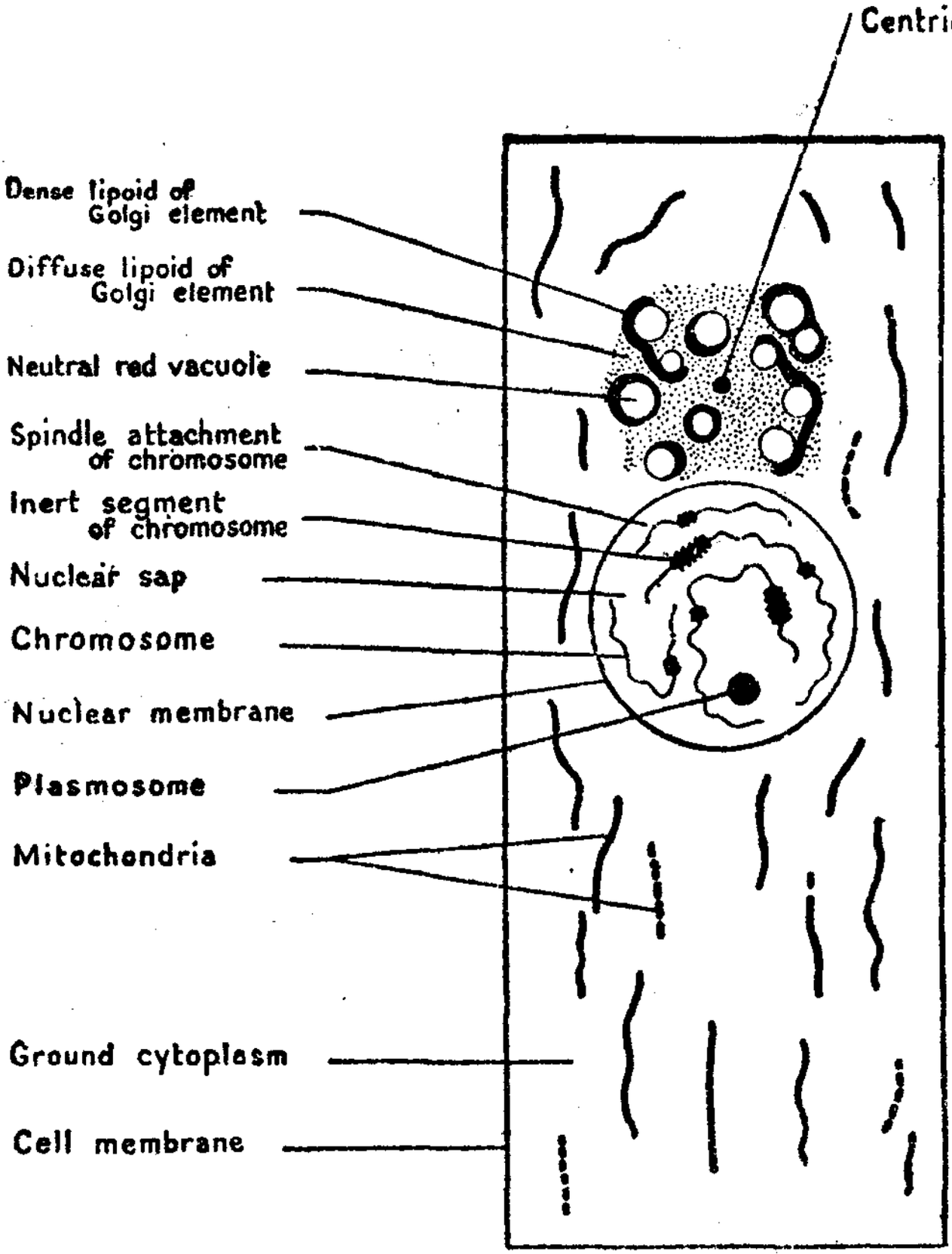

FIG. 1. DIAGRAM OF AN UNDIFFERENTIATED EPITHELIAL CELL, SHOWING WHAT IS THOUGHT TO BE THE USUAL STRUCTURE.

scientific subject, and that no one will think of publishing a new technique without having a full knowledge of the substances used, without considering the possibility of reactions between them, and without giving an account of the results that caused him to choose certain substances and to mix them in certain proportions.

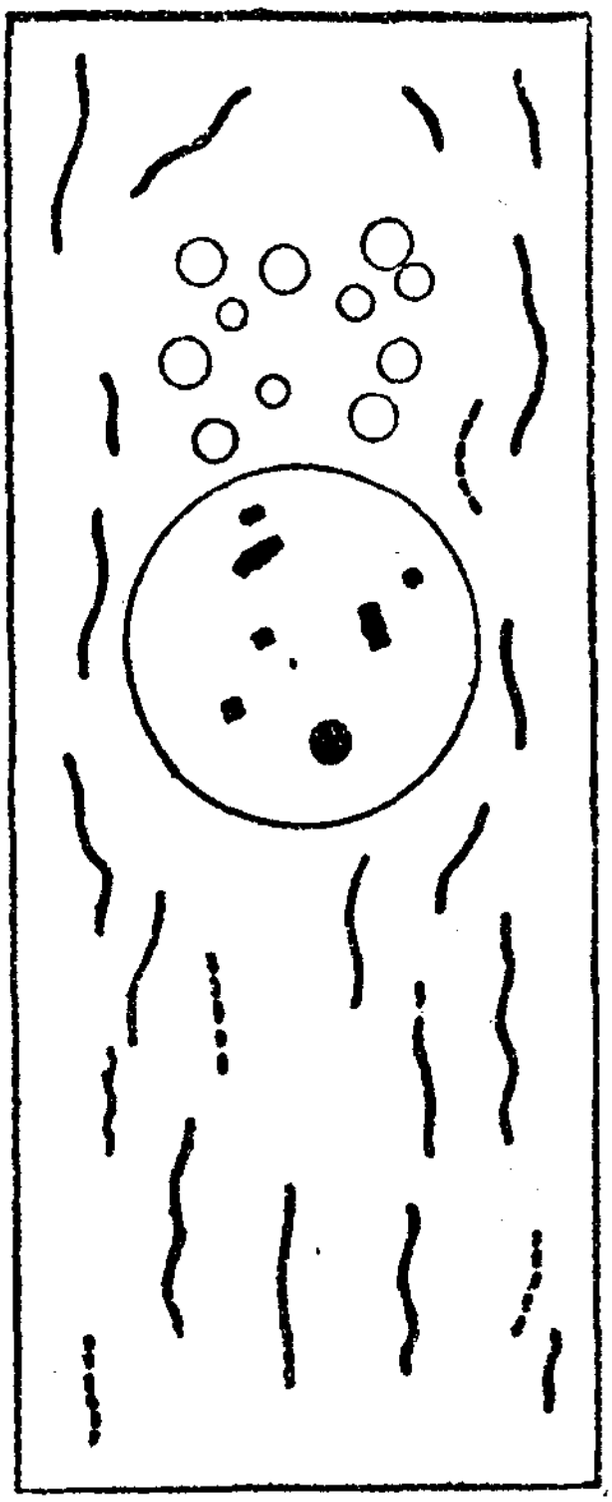

FIG. 2. — DIAGRAM SHOWING WHAT CAN BE SEEN IN AN UNDIFFERENTIATED EPITHELIAL CELL WHILE STILL ALIVE.

The living cell of many-celled animals is very difficult to study, for several reasons. It is usually not possible to separate it from other cells without the help of various substances that kill it, and unless we separate it, we cannot get a good view of it. If we choose a cell which we can observe closely while still alive, we are still confronted with the difficulty that its contents are mostly colourless and transparent, and only distinguishable from one another, if at all, by relatively small differences in the degree to which they refract light. If we use all our patience as well as all our resources in ordinary and dark-ground illumination, we may see the structures shown in

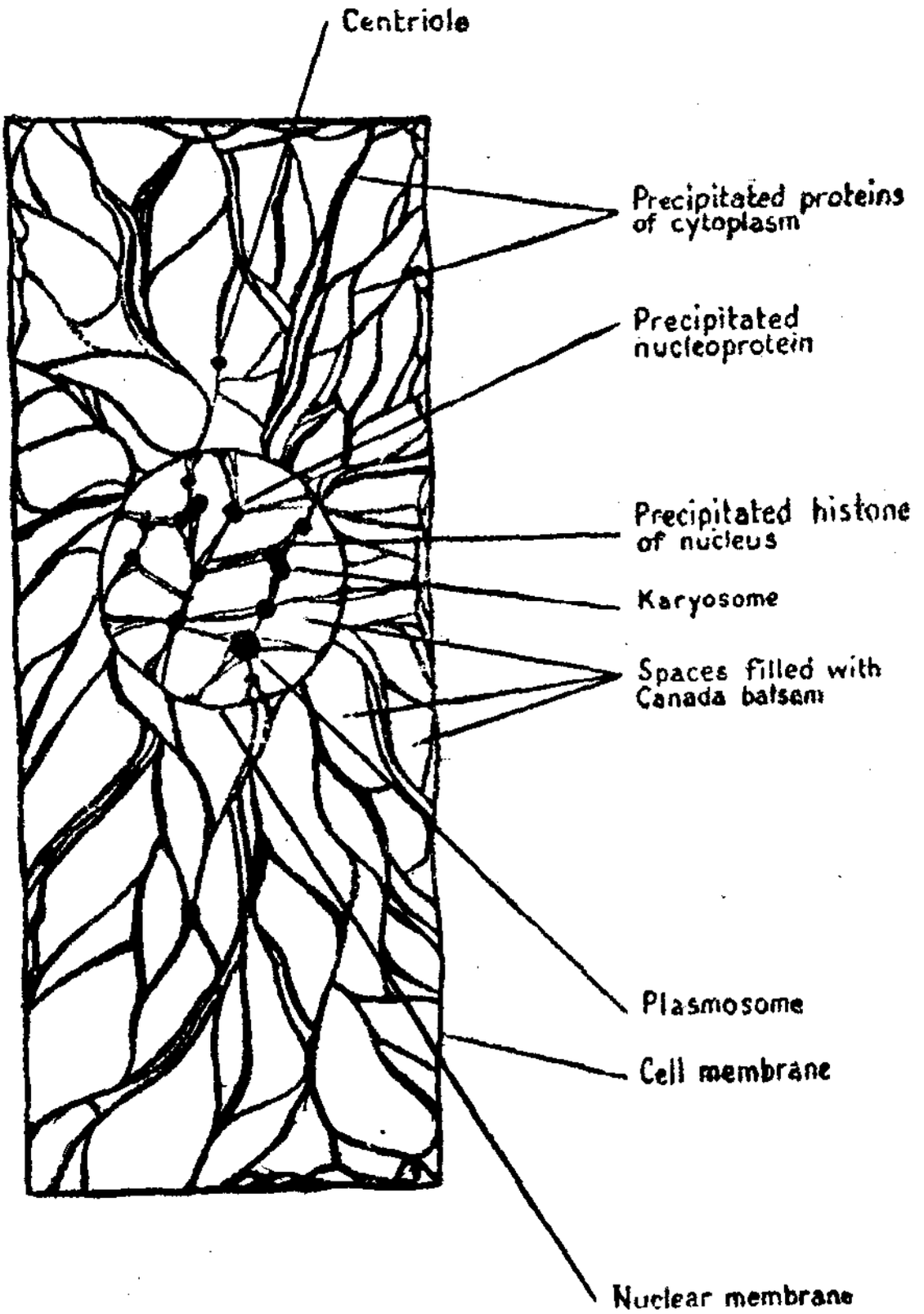

FIG. 3.—DIAGRAM OF AN UNDIFFERENTIATED EPITHELIAL CELL AFTER IT HAS BEEN FIXED BY A PROTEIN-PRECIPITANT, SECTIONED, STAINED AND MOUNTED.

Fig. 2. These structures may be seen while the cell still lies in the body-fluid of the animal from which it was taken, without the use of any fixative or stain. Fig. 1 shows the microscopically-visible objects that may reasonably be supposed to exist in the living cell, and Fig. 3 shows what remains when an ordinary preservative or 'fixative' has acted upon it and a thin section has been cut and stained.

The diagrams show an undifferentiated epithelial cell. The structure of cells can be investigated by the methods described in the succeeding chapters of this book. The chemical composition of the various cell-constituents can be studied by cyto-chemical tests, and inferences can be drawn from our knowledge of the chemistry of the proteins, which, next after water, are the most abundant substances in the cell.

Water makes up some 85% of the cytoplasm, which is fluid but in flowing does not behave like a perfect or 'Newtonian' liquid. The water contains salts and sugars in solution. The kations of the salts are chiefly potassium and magnesium; the predominant anion is phosphate, with some bicarbonate (Lowry, 1943). The salts maintain a *p*H of about 6·8–7·0.

The proteins of the cytoplasm amount to about 8% of it by weight, but the molecules of protein are so huge that there is only one to some 18,000 of water (Sponsler and Bath, 1942). It was first suggested by Meyer in 1928 that the protoplasmic proteins occur in two forms (see Meyer, 1940). On the one hand there are the separate or 'globular' molecules of globulin and albumin. Each molecule is probably associated with water in such a way that the whole ground-cytoplasm forms an emulsion. Each molecule may have as much as 40% of water associated with it and may measure some 50 Å [1] across (Sponsler and Bath, 1942). On the other hand there are thought

[1] 10,000 Ångstrom units (Å) = 1 μ. If lines are less than about 2000 Å apart, they cannot be separately resolved by the microscope.

to be 'structural' or fibrous proteins in the cytoplasm. These are very long molecules, perhaps some 1,000 Å in length (though only 10 Å wide and 4·5 Å thick). As Seifriz (1942 A) well remarks, 'To attempt to satisfy the physical properties of protoplasm with a spherical molecule is rather like asking a weaver of cloth to make his fabric of sand instead of threads.' The fibrous molecules are thought to be joined to one another and to other molecules by chemical bonds of various kinds, so as to make a sort of 'brush-heap' with the long molecules pointing at random in all directions (Frey-Wyssling, 1940). The fibrous proteins provide a structural framework in the cytoplasm and account for its tensile strength and elasticity, and to a large extent for its viscosity. Since the long molecules tend to become parallel when cytoplasm flows, they also account for the birefringence that it shows under certain circumstances. The structural proteins are far less easily removed by solution than the globular, and Banga and Szent-Györgyi (1940) have suggested that it is precisely for this reason that we are relatively ignorant of them: when studying protoplasmic proteins we have unconsciously chosen those that are mobile and hence easily accessible. As Moyer (1942) points out, we must not think of these fibrous proteins of protoplasm as being closely similar to the familiar extracellular fibrous proteins, such as silk; for the fibrous proteins of ordinary cytoplasm are very sensitive to heat and electrolytes. The most resistant of them is 'ellipsin' (Bensley, 1943; Hoerr, 1943), which is not soluble in saline solutions but is extracted by 0·5 N. sodium hydroxide. 'Plasmosin' is regarded by some authors as another structural protein, characterized by insolubility in physiological saline solutions and solubility in 10% sodium chloride. It is claimed by Mirsky and Pollister (1943), however, that this fibrous and sticky substance is nothing else than nucleo-protein, derived from the nucleus and absent from living cytoplasm.

It is thought by some cytologists that lipoids are diffusely dispersed through the cytoplasm, though there is no concrete evidence that this is so. These dispersed lipoids are supposed to be associated with the proteins. Lecithin can exist in a 'Zwitterion' form, with the positive and negative charges 7 Å distant from one another. Sponsler and Bath (1942) point out that this is exactly the distance between adjacent amino-acid residues in proteins. Thus lecithin might become attached to two adjacent, oppositely-charged amino-acid residues. The reader should consult these authors' paper for some very suggestive remarks about the sizes and shapes of the molecules constituting protoplasm.

The cytoplasm is thought to hold a mass of minute lipoid-containing particles or 'microsomes'. Since they are only some 0·05–0·3 μ in diameter, they are as a rule invisible under the microscope, and their size can only be estimated by indirect means; indeed, we cannot be certain that they exist as particles in the living cell. They were centrifugally isolated by Claude (1941, 1943), who describes an isolated mass of them as forming a non-viscous, transparent gel, usually amber in colour on account of the presence of riboflavin. The microsomes may sometimes constitute 25% of the whole of the cytoplasm. They are composed mainly of ribose nucleoproteins and phospholipines.

It is necessary at this point to make a digression on the subject of nucleoproteins. The substance that renders chromosomes so easily stained, and thus gives them their name (= colour-bodies), is nucleic acid. This consists of an equimolecular compound of pentose sugar, phosphoric acid and nitrogenous bases. The nitrogenous bases of nucleic acid are substances allied to uric acid. The pentose sugar of the nucleic acid of the chromosomes is peculiar in that one of the OH groups is replaced by H, so that there are only four oxygen atoms instead of five. The name

'desoxyribose' applied to this sugar refers to this fact, and the nucleic acids containing this sugar are called desoxyribose nucleic acids. In nucleoproteins the nucleic acid is combined with various proteins, but the latter either do not neutralize all the phosphoric acid in the nucleic acid molecule, or else are themselves acidic; for the compound remains acid. (The nucleoproteins of certain spermatozoa provide an exception on account of the strongly basic nature of their proteins.) It is the acidity of nucleoproteins that gives the strong reaction with what are called 'basic' dyes (see pp. 134–9). In some nucleic acids, called ribose nucleic acids, the pentose sugar has the normal complement of five oxygen atoms, and these nucleic acids, curiously enough, neither stain strongly with basic dyes nor give the ordinary histochemical reactions of the nucleic acid of chromosomes. They can be recognized as nucleic acid, however, by their specific absorption of certain wave-lengths in the ultra-violet. The word 'chromatin' was coined by Flemming in 1879 for the substance in nuclei and chromosomes that stains strongly with basic dyes. (The chromosomes themselves had to wait another nine years to get a name.) The old-fashioned name 'chromatin' is still useful, and I shall use it in this book to mean *acidic compounds of desoxyribose nucleic acid with proteins*. It may be remarked, however, that according to some authorities free nucleic acid occurs in the fully-formed chromosomes, and if so, the meaning of the word must be extended to cover uncombined desoxyribose nucleic acid. The expression 'non-chromatic nucleoprotein' is a convenient alternative for ribose nucleoprotein, since it recalls their lack of special affinity for chromatin dyes. The microsomes, then, consist largely of non-chromatic nucleoprotein.

The cytoplasmic substances so far mentioned are invisible on ordinary microscopic examination of the living cell. The methods of colloid chemistry and of

X-ray and ultra-violet analysis, together with differential centrifuging and the freezing-drying technique of Gersh (1932) and his associates, have been necessary to disclose them. We turn now to cytoplasmic constituents that are visible in the living cell under the ordinary microscope.

The cytoplasm is bounded externally by the cell membrane. This is thought to contain a continuous film of lipoid molecules, those next the cell-surface being orientated with the hydrocarbon part turned inwards and the hydrated polar groups outwards, while the molecules next the ground cytoplasm are the other way round, with the polar groups inwards towards the cytoplasm. Protein is adsorbed on the polar groups both on the outer surface of the membrane and on its inner surface, next the cytoplasm. This is the structure ascribed to the cell membrane by Danielli (1942). The protein of the cell membrane is stated to contain ellipsin (Bensley, 1943).

The ground cytoplasm contains three 'elements' or constituents that are thought to be nearly essential for the life of Metazoan cells, because they occur in nearly every cell of many-celled animals and are self-perpetuating. These three elements are the mitochondria, the Golgi element (or 'apparatus'), and the nucleus.

The mitochondria are threads, rods or granules. They are smooth in outline, all of about the same diameter in any one cell, and unbranched. The most striking fact about them is that stages in growth from thinner threads or smaller granules are scarcely ever seen, and this fact, often overlooked or insufficiently stressed, provides the strongest evidence that they are self-perpetuating bodies. They grow in length and multiply by transverse division. A thread sometimes divides into two or resolves itself into many short rods or granules of the same diameter.

Altmann (1894) was the first to make a careful

study of mitochondria and to devise a special method for showing them, though they had been seen by others long before. He made the mistake of considering them as 'Elementarorganismen', that is, as simple organisms living in the cytoplasm. It was Benda (1898) who first recognized mitochondria as a cellular element, an almost universal cell-constituent. He tells us (1902) that when he had found mitochondria in different kinds of cells of various animals and finally in blastomeres and Protozoa, and had shown that they have an existence separate from that of other cell-organs and remain as isolated bodies during mitosis, he came to the conclusion that the so-called granulation of cytoplasm represented a specific component of the animal cell. It was he who first gave them the name of mitochondria at a meeting of the Berlin Physiological Society on 29 July, 1898 (Benda, 1898). He refers to the 'Körnern oder richtiger: Körner-faden' that he first studied in the spermatids of the mouse and Phalangista and subsequently found in many cells of many different classes of animals. He mentions that he has evolved a special method for staining them. 'I should like provisionally to reserve for them,' he says, 'a special position as mitochondria, a position that I shall substantiate in further works.' This is the first use of the word mitochondria. Benda kept his promise in many writings, including the already-mentioned 1902 paper, in which he translates the word mitochondria as 'Fadenkörner' (thread-granules). The cytoplasm of the most diverse cells does in fact contain threads or granules which may be shown by special methods and which have certain definable properties, and the thread-like and granular forms are known in certain cases to be interchangeable. Benda's word 'mitochondria' is therefore both convenient and accurate. It is unfortunate that French cytologists, with most ungallic illogic, call only the *granular* form by the name mitochondria and use 'chondriosomes' (granule-