CYTOCHEMISTRY

A Critical Approach

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To the Memory of
Sir Jack Drummond

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CHAPTER I

INTRODUCTION

Cytochemistry is an almost undeveloped branch of biology. It is only comparatively recently that a vigorous attempt has been made to solve the problems which are involved in cytochemical studies. Consequently, anything in the nature of a textbook on cytochemistry is premature. There is an insufficient body of agreed facts for it to be possible to write a textbook which would set forth a body of methods and knowledge which would be agreed to without question by the great majority of workers in this field. I wish therefore to make it clear that this book is not intended in any way as a textbook. It is very largely a record of experiments which I have carried out myself, or which have been carried out by other research workers with whom I have been closely associated. Any of the individual methods given here may well be superseded in a few years. I do, however, hope that a good deal of what is recorded here may be regarded as in the nature of a blueprint for future developments in cytochemistry. The general endeavour which is contained in this work, namely, the development of rigorous methods, is a key matter without which cytochemistry is a futile study.

Cytochemistry is pre-eminently a field which calls for a team of workers. The reason for this is that it demands a high standard of knowledge in each of the fields of biology, chemistry, and physics. Unfortunately, many of the methods which have been developed so far appear at first glance to be so simple that the necessity for rigorous treatment has largely tended to be ignored. There have, of course, been a number of outstanding instances of compliance with the experimental criteria which are required: this is obvious to all who know of the work of Feulgen, of Caspersson, or of Commoner. But the general tendency has been to suppose that anyone who can cut a tissue section and make up a standard solution is competent to carry out cytochemical investigations. Nothing could be further from the truth. Indeed,

it may almost be said that a good training in histology is one of the worst backgrounds possible for cytochemical work. In the type of histology and cytology which is based on showing optimal fixation, optimal staining, and optimal methods of treatment with stains, a tremendous amount depends upon the judgment of the investigator as to what he wishes to stain and just how he wishes to see it. In some sense, this type of histology and cytology is an art. On the other hand, the object of the cytochemist is to use fixatives and cytochemical methods in such a way that a specific and exact treatment is applied to a tissue, in a manner which is predetermined by the physical properties of the specimen and a precise programme of chemical treatment. During the carrying out of this programme, the judgment of the investigator must be suspended in the interest of maintaining the precision of treatment. It is only after the full processes of physical and chemical treatment have been carried through that the investigator can allow his opinion to operate. I feel obliged to add that the standards of cleanliness of reagents which are frequently adequate for classical histology are, in my experience, frequently inadequate for cytochemical purposes. So much is this so that, whenever I hear that an investigator has failed to obtain success with a cytochemical method, my first piece of advice is always that he should throw away every reagent that he has been using and make up a new set of reagents; it is surprising how frequently this elementary step is successful.

In the second half of the nineteenth century there was a considerable wave of interest in cytology and histology; this was enabled to come to fruition by the discovery of synthetic dyes which could be used for staining fixed material. It is quite clear from the literature of this period that the investigators were equally concerned to obtain information about both the physiological and the chemical organisation of protoplasm. Their success, however, was very largely limited to the morphological and physiological side: there was an almost complete failure on the chemical level. Amongst the reasons for this was the fact that affinity for a given stain is only to a limited degree determined by the detailed chemical constitution of the material being stained. It is much more markedly determined by the physical properties of the material. Difficulties were also en-

countered with artefacts due to fixation. This matter was brought to a head by W. B. Hardy and A. Fischer in two publications in 1899. These two investigators had a much clearer understanding of the physical basis of cytological techniques than had most of the previous workers in this field. They showed that the details of the procedure of fixation, of the nature of the fixative, and the technique of staining, have just as profound an effect upon the final picture as has the initial chemical composition of the material being stained. They also showed that many of the fixatives in common use were giving rise to structures which, in fact, did not occur in the living cells, but were precipitation artefacts. At this time, neither the chemistry nor the physics of the systems involved in cytology were sufficiently understood for it to be possible to cope with the problems demonstrated by Hardy and Fischer. Consequently, by 1910 the wave of interest in the study of fixed preparations had very largely lost its momentum so far as pioneering investigations were concerned. The pioneering investigators instead turned to working on living cells almost exclusively. There was, in consequence, a very rapid development of experimental cytology.

Between 1910 and 1935 remarkable progress was made in the field of experimental cytology. Some phenomena were so successfully analyzed that the chief physico-chemical factors involved could be detailed, and given an approximate mathematical treatment. However, the result of this successful investigation was that many of the working hypotheses formulated by the experimental cytologists postulated specific cytochemical organisations in particular parts of cells. Such postulates were found in many widely diverse fields, e.g., ciliary and amoeboid movement, muscular contraction, secretion, and the action of genes. The postulated cytochemical organisations lay so fundamentally at the heart of the mechanisms proposed that further progress was bound to become increasingly limited in every field, unless further advances were possible in cytochemistry. It is not surprising, therefore, to find that from 1935 onwards there was a renewed wave of interest in cytochemistry. Landmarks in this new wave of interest were the publication of Lison's book "Histochimie Animale," and the papers of Feulgen, Caspersson, Gomori and Takamatsu, Linderstrøm-Lang, and

Holter, who blazed new trails into the wilderness of cellular chemistry. It has, however, been somewhat unfortunate that many of the lessons which can be learned from the work of Hardy and Fischer and others have been overlooked in more recent studies.

In the development of rigorous methods of investigation in cytochemistry, it is necessary to look at each problem from three points of view: as a chemist, as a physicist, and as a biologist. From the point of view of the physicist, the main problems arise from diffusion and adsorption artefacts, from estimating errors which may arise from the state of aggregation of the substance which is being studied, from the degree of molecular orientation of the substance, and from the scattering of light within a specimen. No qualitative study can be regarded as satisfactory which does not involve the elimination of diffusion and adsorption artefacts. Correspondingly, no quantitative study can be regarded as satisfactory unless the degree of aggregation of molecules in the specimen, the orientation of the molecules in the specimen, and the scattering of light within the specimen are properly taken into consideration.

From the chemist's point of view the main problems are to use methods which are of sufficient specificity and which shall be quantitative and accurate to a known extent. A further practical problem which frequently arises is to find methods which will be to a sufficient degree inert, i.e., which do not damage the specimen. The problem of chemical specificity is a particularly difficult one. The reason for this is that chemical reactions are not carried out by molecules as a whole. They are usually carried out by a very small number of atoms in a molecule. They are often affected to a marked degree by a rather larger number of neighbouring atoms; but the greater part of the molecule may well have no effect on whether a particular reaction occurs or not. Thus the specificity of a chemical reaction is limited to supplying information as to whether a particular chemical group is present in a particular part of a cell. Information as to the nature of some of the other neighbouring groups in the same molecule may sometimes be obtained by studying the rate at which a chemical reaction proceeds. But it is impossible to identify the whole of any molecules, other than the simplest, by carrying out chemical reactions. Thus, in

the well-known Feulgen reaction which is commonly said to give localization of deoxyribonucleic acid, we have in fact a method which probably indicates a linkage between deoxy sugar and any other group which one can split off from the glycoside linkage at roughly the same rate as are the purines. Thus the Feulgen reaction, in fact, gives us information which is limited to telling us that deoxy sugar is present in the specimen and that it is in glycosidic linkage with a substance which can be split away by acid hydrolysis at the same rate as are certain purines. The Feulgen reaction does not tell us whether the substance which is split off is a purine, nor does it tell us whether the sugar is linked through phosphate bonds to other similar units so as to complete a nucleic acid. In a similar way, when the ultraviolet spectrum of a specimen is studied, one can readily detect the presence of a substance absorbing in the same region of the spectrum as the purines and pyrimidines. There are, of course, other substances which absorb in this region. A notable example, recently reported by Chayen (1952), is ascorbic acid. Thus, in a material in which ascorbic acid may occur, it is necessary to take steps to differentiate between ascorbic acid and purine groups. When all the necessary elimination of this type has been done, we can perhaps be certain that in a particular part of a specimen there is purine or pyrimidine. But it has not so far proved possible from spectrophotometric studies to determine whether the purine or pyrimidine is present as part of a nucleic acid molecule, or whether it is present in some other form, e.g., linked directly to a protein. It is notable in this connection that Panijel has recently found a protein in *Ascaris* sperm which has the same absorption spectrum as nucleic acid, containing a considerable proportion of purine, but no phosphorus.

The problems which arise from the biologist's point of view are somewhat different in nature and are less readily defined. They must, however, involve a constant awareness of the fact that an animal, and a cell, cannot be dissociated from its environment. Due respect must be paid to such principles as those of homology and analogy, and at the same time it is necessary to maintain a more rigid guard against the acceptance of generalisations than is usually necessary in the fields of physics and chemistry, owing to the fact that variables on the purely biologi-

cal side are far more easily underestimated than otherwise. As examples of some of the problems which affect one from the biological point of view, may be mentioned fixation and diet. It is doubtful whether any method of fixation other than freeze-drying is really adequate for cytochemical purposes. Then, as far as diet is concerned, it will be a source of amazement to future generations to discover in how few of the papers on cytochemistry the diet of the animals used is at all defined. Yet the cytochemical pattern in organs such as the liver and intestine is astonishingly dependent upon diet. As another example, may be mentioned the work of Dr. H. Mugard (1953), who has recently shown that the cytoplasm of the ciliate *Ophryoglena atra* which has not recently fed is apparently free from the enzyme alkaline phosphatase: yet within a few seconds of the formation of a food vacuole a high concentration of phosphatase is present in the cytoplasm. For particular studies it will no doubt be equally important to define the age of the animal under investigation, the time of taking the specimen, the state of hormone activity, and other biological variables, before a generalisation on the cytochemical level may safely be embarked upon.

Before passing on to consider the fields in which I have played some part in developing techniques, a number of techniques will be considered critically, since by so doing it is possible to see many of the major hazards which exist in the field of cytochemistry, and which it has been my endeavour to avoid.

Centrifugal Stratification, etc.

In these techniques the common principle is, by centrifugation or other methods, to separate parts of cells which differ from one another in their physical characteristics. The most usual approach is to stratify a cell by centrifugation, to separate the strata, and then examine the distribution of various substances in the different strata. In the hands of Holter and of Shapiro, this work has provided valuable information about the distribution of certain substances, particularly enzymes, in large cells such as amoebae and echinoderm eggs. There are, however, some very marked limitations to techniques of this type. Their use is usually limited to large individual cells:

cells in tissues cannot be studied readily. Then the methods of manipulation are not devoid of action upon cell fractions which are under study. For example, Shapiro found that when sea urchin eggs are centrifuged, first so as to cause stratification and then so as to cause the cell to divide into two halves—one light and one heavy—the respiration of the two fragments so formed is greater than that of the initial intact egg. It therefore follows that in an experiment of this type either there has been a change in the physico-chemical organisation of the enzyme systems of the cell, or else there has been synthetic activity on the part of the cell, resulting in the formation of more respiratory enzymes. Whichever of these changes may have taken place, it is clear that the final condition, as revealed by the cell fragments, cannot be taken as a close guide to what was happening in the intact egg. In this connection, Holter remarks, "The only conclusions to be drawn from such experiments are those based on the distributions of *substances,* ··· [which] ··· permit, of course, only indirect conclusions with regard to physiological activity." In any experiments involving the destruction of the known relationships between cellular entities, as is the case in stratification, any deduction is dubious unless it is established that the procedure does not lead to synthesis or to destruction of chemical components. It is obligatory in such experiments to establish the lack of such synthesis or degradation.

Maceration Procedures

Amongst the most common techniques employed today, particularly by biochemists seeking to make a contribution to cytology, are techniques involving the disintegration of cells into fragments, and fractionation of the fragments so formed. The debris formed by maceration is commonly centrifuged at various speeds so as to isolate fragments with different sedimentation rates. It is hoped that methods of this type will isolate granules, mitochondria, nuclei, and chromosomes, in a condition which is closely similar to, if not identical with, the state of those bodies in the intact cells. It would undoubtedly be of the greatest value if it were true that cell organs could be isolated in this way. But so far there has been an almost

complete lack of proof that the bodies isolated are in the same condition as in the intact cells.

It would, indeed, be very surprising if there were not many and dramatic changes in the organisation and composition of both nuclear and cytoplasmic bodies as the result of maceration and addition of the various solutions which are used in fractionation. The nucleus and the cytoplasm are both very complex colloidal systems. Studies by Chambers and others on cell nuclei have shown that when the nucleus is removed from the cell by microdissection it commonly either sets into a gel or dissolves: in either case a profound change occurs as soon as the nucleus is removed from its normal environment. De Fonbrune, and Lorch and Danielli have found that, although a nucleus may readily be transferred from one cell to another in a viable condition provided that it does not come into contact with the environment of the cell, a few seconds' contact with the environment is sufficient to destroy the viability of the nucleus. Dr. Dounce has informed me that he has compared the composition of nuclei isolated from macerated cells by centrifugation in non-aqueous solvents with nuclei isolated by centrifugation in aqueous solvents. The nuclei from the non-aqueous solvents contain almost twice as much material as do those from the aqueous solvents. From these few remarks it is quite clear that one should anticipate profound changes in the organisation of the nucleus when it is removed from its normal environment, and that these changes must include diffusion of substances out of the nucleus, and probably also diffusion of substances into the nucleus. It should be the first responsibility of the investigator to ascertain the extent to which morphological changes and diffusion artefacts are involved in these isolation techniques. It seems probable that at the present time the only work of this type, i.e., on isolated nuclei, which is reliable is that of Brachet and of Callan on some of the properties of the whole nuclei of amphibian oocytes, which were isolated by microdissection techniques.

It is probable that the organisation of the cytoplasmic components is just as labile as that of the nucleus, if not more so. Often in relatively simple protein systems, such as blood plasma, addition of foreign materials causes profound reor-

ganisation. For example, addition of salts may vary the proportion of albumin and globulin found on electrophoresis of plasma. Somewhat similar effects are found on simply adding water to plasma, and a not immoderate addition of water causes precipitation of part of the globulin. It is difficult to see how experiments on the isolation of granules and other small cell organs can be taken at their face value unless it is rigorously demonstrated that their composition is unchanged by the isolation procedure.

Often, if the composition of individual granules is identical or closely similar to the granules in the living cells, there still remain many difficulties in interpretation of experiments on the mass isolation of granules—mitochondria, nuclei, etc. The reason for this is that it is practically impossible, except in a small minority of tissues, to obtain a homogeneous concentration of cells. In the first place, almost all tissues contain, in addition to the typical cellular component, cells of the vascular system, connective tissue cells, and white and red blood cells. Moreover, often the cells of the same type, e.g., hepatic cells, are not of identical chemical composition in closely adjacent parts of an organ. Consequently, it does not follow that a biochemical pattern observed in a particular group of granules is identical with, or even similar to, that of any individual cell type. This can be very simply demonstrated in the case of hepatic cells. Long-chain aldehyde (Feulgen's plasmal), ribonucleic acid, and alkaline phosphatase will occur in the same granule fraction obtained from liver. It would, therefore, be natural to suppose that these three components are bound together in the same granule and may even cooperate in carrying out certain biochemical functions. When, however, the distribution of these three components is studied in tissue sections, it is found that each of them is present to a significant concentration only in some of the hepatic cells. Some hepatic cells may be practically free of any one of these components, some may contain one component, some, two components, and some, three components in significant quantities. If, therefore, all three substances are present on a granule of the same size in the living cells, then it appears that some of the granules contain none of the substances, some, one of the substances, some, two, and some,

three. It is thus quite misleading to suppose that all the granules must have all three of these substances, as has been deduced from the procedure of isolation of granules.

It should also be emphasized here that the phenomenon of lack of biological identity of cells, even of the same cell type, illustrates a general weakness of deductions drawn from biochemical studies of extracts. As a general rule, no tissue can be regarded as biologically homogeneous, and deductions made on the biological level after disintegration of tissues must normally be treated with considerable reserve. Only in the case of relatively homogeneous cell preparations, such as those of yeast, bacteria, ova and sperm, can such studies be regarded as referring to a single chemically homogeneous cell population.

In maceration experiments, it is also inevitable that activation and inactivation of enzymes will occur to a degree which may often present formidable difficulties. How this problem can be coped with is not at all clear. There is also the difficulty described by Marjorie Stevenson, that one is tempted to suppose that all enzymes found in a cell must have a function. Dr. Stevenson was inclined to think this must be the case until she found an enzyme in bacteria which could act upon chlorate. She could not believe that this activity could be of any value to the cell since chlorate never appears in its normal environment. One hesitates to agree entirely with this argument: it may well be that the enzyme which acts upon chlorate also acts upon some other substrate which is normally found in the environment, or which from time to time occurs in the environment. And another difficulty in the interpretation of enzyme activities is to know whether any individual enzyme is functioning in connection with a particular physiological activity which is under observation. Indeed, it does not necessarily follow that all the enzymes found in a cell are necessarily functioning at the same time. It seems quite possible that some enzymes may be present in cells at all times, but have a function to fulfill only at exceptional periods in the life of the cell.

When all these experimental hazards and difficulties of interpretation are considered, it must be clear that it will be a long time yet before the results of maceration procedures can be either evaluated or interpreted.

THE SILVER NITRATE-ACETIC ACID TEST FOR ASCORBIC ACID

When tissue is treated by silver nitrate dissolved in acetic acid, it is commonly found that deposits of silver are formed due to reduction of the silver nitrate. It has been claimed that reduction of silver under these conditions is a specific test for ascorbic acid and also that the localization of the silver deposits is a close guide to the localization of ascorbic acid. However, owing to the diffusion factors involved, these conclusions can hardly be true; indeed this system is an excellent example of the complications which are bound to ensue when such chemical studies are attempted on small molecules. Molecules of low molecular weight, such as ascorbic acid, silver nitrate, and probably also the initial reaction product of ascorbic acid with silver nitrate, are highly diffusible. It is thus easy to say much in theory, but it is impossible to prove the precise localization of ascorbic acid by this method. As the silver nitrate-acetic acid mixture diffuses into cells a mixing zone will be established somewhere close to the cell wall in which ascorbic acid and silver nitrate will react. As the reaction proceeds fresh silver nitrate and fresh ascorbic acid will be recruited into the mixing zone until the ascorbic acid supply is exhausted. This interaction in the mixing zone is inevitable with two such highly diffusible substances. Consequently, if ascorbic acid exists in cells as such, it can only be demonstrated in the mixing zone, which is an artefact of fixation. In fact, the silver which is formed by reduction in this procedure is not found in the mixing zone but attached to mitochondria, etc. It is thus clear that either one of three things must be true. The reaction may be truly occurring at the surface of the mitochondria; but if this is true, it is not with free ascorbic acid that the reaction is occurring but a substance bound to the mitochondria so as to render it non-diffusible. An alternative explanation is that the technique does involve a reaction of silver with free ascorbic acid but that the results of the reaction appear adsorbed on surfaces which have a high affinity for the reduced silver and which bear no relationship to the distribution of ascorbic acid in the living cell. A second alternative is that diffusible ascorbic acid is released from the interior of the mitochondria by fix-

ation, and precipitates silver in a mixing zone at the surface of the mitochondria.

Techniques for Cytochemical Demonstration of Enzymes

These techniques usually involve carrying out an enzyme reaction with the intrinsic enzymes of a tissue section or smear, etc., under such conditions that one of the products of the reaction is precipitated. Before results of this type can be evaluated, it is necessary to have answers to a number of questions. These are: (*a*) How much of the enzyme is destroyed before and during the cytochemical procedure? If destruction occurs, does it occur to an equal degree at all sites or does it occur selectively at certain sites? (*b*) Is the insoluble reaction product precipitated at the actual site of enzyme reaction, or is it precipitated at sites which have a very high affinity for the reaction product? (*c*) Is the enzyme in the specimen used for cytochemical study in its physiological position in the material, or has it been redistributed by diffusion processes? Studies which do not provide answers to these questions must be discarded. It is, no doubt, true that some of the methods now used to study the distribution of enzymes do, in fact, demonstrate the correct site of the enzyme; it is equally true that there are other instances in which the information provided is greatly misleading.

The Feulgen Technique for Nucleic Acid

In this technique tissues are heated at 60° with normal hydrochloric acid for 5 minutes or more, the precise time varying with the fixative which is used and the nature of the specimen. This procedure splits off purine from the deoxy sugar nucleic acid, so that, when the material is subsequently treated with reduced fuchsin, a violet Schiff's base is formed. The procedure of hydrolysis, however, tends also to make nucleic acids diffusible, probably by reducing the molecular weight, as was suggested by Stedman and Stedman. That this was likely to be the case has been implicit in results known for many years. For example, Bauer showed that, when a fixative like formaldehyde is used, or acetic alcohol, the intensity of the reaction reaches a peak after, say, 5 or 10 minutes of hydrolysis. Further hydrolysis