Quantitative Cytochemistry and its Applications

Edited by J. R. Pattison
L. Bitensky and J. Chayen

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PREFACE

Histochemistry has developed as a branch of histopathology and tends to emphasize the sub-cellular localization of stain. The origins and aims of cytochemistry are quite different. Basically, the procedures used in cytochemistry are very similar to those used in biochemistry, the most important difference being that, in cytochemistry, the coloured reaction product is precipitated within active cells. This has a number of advantages. Firstly, biochemical activity of cells can be related to histological appearance. Thus, with tissues containing a variety of cell types, biochemical functions of a single cell type can be studied with the cells in a normal structural, and presumably functional, relationship with the other cell populations. Alternatively, cytochemistry permits the detection of functional differences between cells such as hepatocytes which might otherwise appear to be a single cell population. Again, preservation of the normal architecture of a tissue appears to be critically important in relating differences in function to localization of cells within an organ. The second advantage of cytochemistry is that it is a non-disruptive technique. Thus the biochemical functions of sub-cellular organelles and their membranes can be studied without isolating the organelles into a foreign medium, a procedure which, in itself, will almost certainly affect the reactions under study. Moreover there is increasing evidence that the cell functions as a unit and therefore the functions of sub-cellular organelles should be studied with the organelles in their natural setting. All the above advantages will tend to be obscured by conventional biochemistry in which homogenization of tissue is a common practice.

The essential basis of *quantitative* cytochemistry is the ability to measure the reaction product. This can be done with a micro-spectrophotometer (or microdensitometer) which is simply a spectrophotometer built around a microscope so that the extra magnification allows the biological cell to replace the cuvette of the conventional spectrophotometer. The problem with measuring reaction products inside cells is that the precipitates are not

homogeneously distributed in the cell. This can be overcome by scanning an area of optically heterogeneous precipitate with a small spot, measuring the extinction of each point and then integrating the extinction for the entire area. A true mean integrated extinction for the area will result if the scanning spot is smaller than the smallest inhomogeneity and the instrument most commonly used is the Vickers M85 scanning and integrating microdensitometer. Granted that such instruments are available, then a further basic advantage of quantitative cytochemistry becomes apparent. Since the biochemical activity of an individual cell or part of a cell is being measured, the sensitivity of quantitative cytochemistry is increased by a factor of about one million compared with conventional biochemistry. In the field of bioassay of polypeptide hormones, the cytochemical techniques are a thousand times more sensitive than even radio-immunoassay.

During the past decade, detailed methods of quantitative cytochemistry have been devised and validated. Consequently, the basic methods of quantitative cytochemistry are well established in many centres in different parts of the world. Gradually, as results appeared, it became apparent that quantitative cytochemistry was yielding useful answers to applied problems, answers which could not be gained or could only be hinted at by conventional techniques. Thus it appeared to two of us (LB and JC) that it was time to arrange a meeting to discuss the applications of quantitative cytochemistry and the results obtained since previous meetings had tended to concentrate on details of the methods used. In its turn, the Council of the Section of Pathology of the Royal Society of Medicine, London, was impressed by the range of applications of quantitative cytochemistry and the subject appeared to provide the sort of interdisciplinary meeting which is the aim of the Section. Accordingly, the Section of Pathology supported a two day international meeting on 'Quantitative Cytochemistry and its applications' which was held at the Royal Society of Medicine, London on November 13th and 14th 1978. The meeting generated intense interest and proved to be a great success. Therefore it seemed desirable to produce a published work in relation to the meeting. The current volume has taken the same title as the meeting and has continued to concentrate on emphasizing the applications of cytochemistry to a wide variety of problems, and on the results produced.

It is essential that we acknowledge the debts we owe to many people who assisted in the staging of the meeting and the production of this book. The Section of Pathology of the Royal Society of

Medicine were given generous financial support for the meeting by Vickers Instruments Ltd.; Sterling-Winthrop; Boehringer Corporation; Organon International; Cordis Laboratories Inc.; Syntex Research Centre; Sigma Chemical Company Ltd. and May & Baker Ltd. The Officers of the Royal Society of Medicine were generous in their support throughout and in particular we would like to acknowledge the experience and enthusiastic help of Mrs. E. Coley, the Sections Officer. Finally, we thank our secretaries, Mrs. D. Blewer and Miss P. Lamb without whose skilled and dedicated help the editing of this volume would have been exceedingly difficult.

J.R. Pattison
Lucille Bitensky
J. Chayen

CONTENTS

A REVIEW OF NUCLEIC ACID CYTOPHOTOMETRY IN GENERAL PATHOLOGY

W. SANDRITTER

Background

Over the past twenty years cytophotometry has become increasingly applied to medical research problems. Without doubt, one of the pioneers of nucleic acid cytophotometry was Miescher, in Basle, who in 1870 isolated nucleic acids from salmon sperm. Another was the physicist Köhler in Zeiss-Jena: in 1904 he constructed a monochromatic microscope objective capable of focussing light of 257 nm and described the absorption of ultraviolet light by the nuclei and cytoplasm of cells; however, he did not recognize the importance of these findings. It was Dheré, in Paris in the same year, who first demonstrated that nucleic acids absorb ultraviolet light. Some thirty years later Caspersson [1936] developed the basic principles of cytophotometry by combining the Köhler microscope and Dheré's observations. In the meanwhile Feulgen's classical chemical, and later cellular studies [Feulgen *et al.*, 1924] produced a chromogenic reaction for DNA which allowed the use of visible-light cytophotometry for measuring the DNA-content of nuclei [Vendrely, 1955]. Nowadays cytophotometry is used mainly for measuring the content per cell of DNA and of RNA but methods exist for simultaneous reactions for protein and for DNA [Lederer and Sandritter, 1967].

Techniques

A cytophotometer, or a microdensitometer, is a simple instrument which accurately quantifies in relative, arbitrary units, the amount of DNA, for example visualized by the Feulgen reaction, in individual nuclei. The basis of the assessment of the results depends on the cell-cycle [as discussed by Wickramasinghe, this volume]. Many nuclei are measured and the results are plotted as a frequency diagram, with

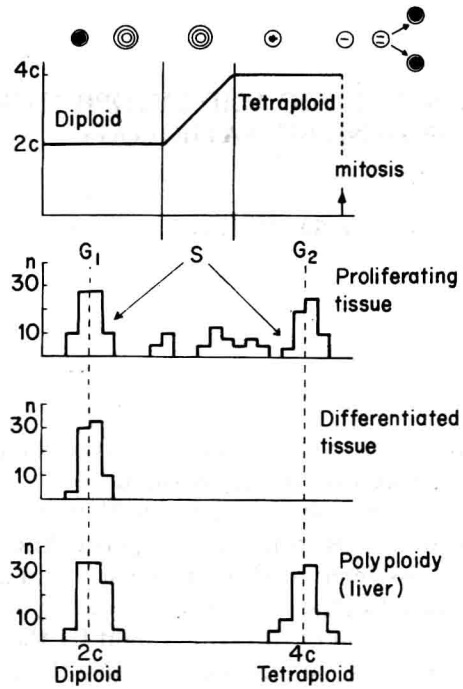

Fig. 1 *Top graph:* The DNA-cycle in interphase and leading to mitosis. *Histograms:* The population-histogram of proliferating tissue shows the 2c and 4c populations, corresponding to cells at G_1 and G_2 respectively, with cells having intermediate values (corresponding to the S-phase). The population-histograms of differentiated tissue show only the 2c or the 2c and 4c classes, depending on the ploidy of the cells.

the DNA-content along the abscissa and the number of nuclei showing a particular content of DNA on the ordinate (Fig. 1).

Basically we can distinguish three patterns of DNA-distribution (Fig. 1). In differentiated tissues we may find cells with only the diploid (2c) content of DNA; in polyploid organs, where both diploid and tetraploid cells occur, two peaks of DNA corresponding to the 2c and 4c amounts are apparent; in proliferating tissues some nuclei will have DNA-values intermediate between the 2c and 4c amounts because these cells are in the S-phase. However, it is important to recognize that cytophotometry alone does not distinguish between cells in S-phase and those which, like malignant tumours, have an aneuploid amount of DNA [also see Wickramasinghe, this volume].

Deviations from normal ploidy

Particularly in human malignant tumours, the diploid DNA-distribution pattern may be disturbed by aneuploidization or in normal cells by polyploidization [Wickramasinghe, this volume]. The mechanism by which this occurs is not yet fully understood. In organs with low rates of proliferation, such as human liver, tendon, salivary gland or heart muscle, in which there is virtually no cell division after birth, growth regulatory mechanisms and other factors, such as hyperfunction, induce the synthesis of DNA which is not followed by mitosis. Three examples of polyploidization will be selected:

i) During the first twelve days after birth all the cells of the *parotid gland* have the diploid amount of DNA but thereafter the number of cells with the tetraploid (4c) and even octaploid amounts of DNA increases steadily. We believe that ageing is one factor that induces polyploidization, but other mechanisms may also be involved.

ii) Virtually the same process of polyploidization with advancing age occurs in the *liver*, producing 2c, 4c and 8c values per nucleus in normal liver cells. But mechanisms other than ageing can be responsible for such changes. Feeding mice with phenobarbital, which

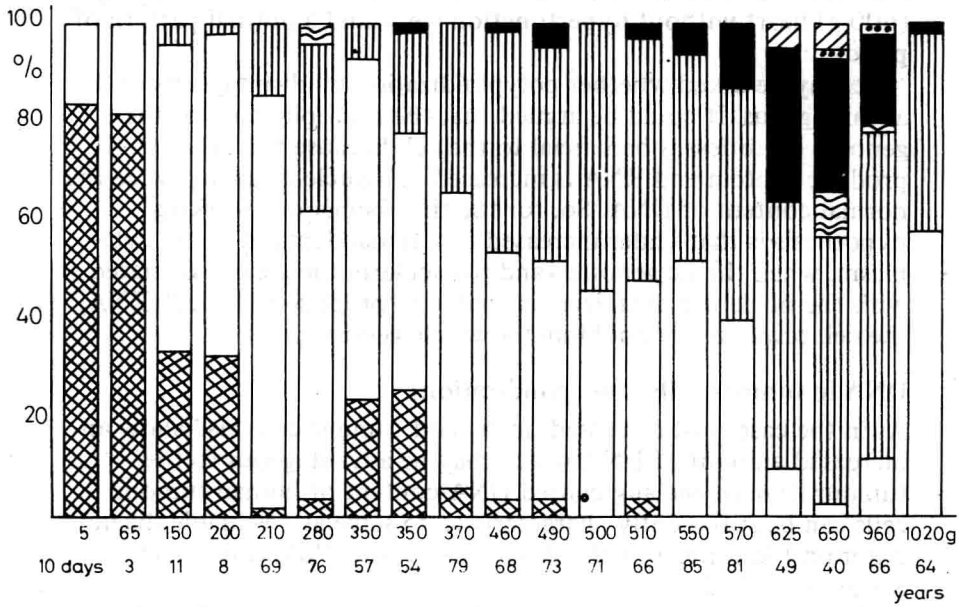

Fig. 2 Relationship between the weight (g) of the heart at post-mortem, the age of the patient, and the proportion of the cells in the left ventricle which show the various contents of DNA. ⊠ 2c; □ 4c; ▥ 8c; ≋ 12c; ■ 16c; ⊡ 24c; ▨ 32c.

induces an increase of the smooth endoplasmic reticulum and of microsomal enzymes [cf. Bridges, and also Chayen *et al.*, this volume] results in an increase of the higher ploidy classes of liver nuclei with more 4c, 8c and 16c nuclei and less 2c nuclei [Böhm *et al.*, 1976]. Yet this process is reversed within ten days of stopping the drug. From these observations we conclude that this polyploidization is a response to an increased functional demand of the cytoplasm.

iii) Our findings in human *heart muscle* [Sandritter and Scomazzoni, 1964; Sandritter and Adler, 1971] support this concept that polyploidization is related to functional demand (Fig. 2).

Up to 12 years of age the cells of heart muscle have the 2c content of DNA; thereafter the number of 4c nuclei increases. In hypertrophic hearts the number of polyploid cells increased with nuclei having the 32c value of DNA (Fig. 2). Thus the polyploidization is concomitant with hyperfunction, and this view is supported by measurements of DNA-content in the hypertrophied regions of the hearts of infants with congenital heart failure. For example, in the right ventricle of a Fallot's tetralogy in a 4-month-old girl, where normally all the cells would have the 2c amount of DNA, we found a great number of 4c and 8c cells. Conversely, in a splanchnomegalic (600 g) heart without hyperfunction we found a normal pattern of ploidy.

It may be asked whether polyploidization is advantageous or disadvantageous. It could be argued that the multiplication of the genome doubtless is beneficial to the cell because the capacity to produce protein and RNA is increased in a nucleus having twice the normal content of DNA. So, for the time being, our working hypothesis is that under increased functional demand in the cytoplasm, when the 2c genome (and particularly the genes associated with the nucleolar organizer) is unable to produce sufficient RNA, the cell responds by doubling the whole genome.

DNA in tumour cells (aneuploidization)

As in the cases just discussed, malignant tumour cells also show an increased amount of DNA which may be advantageous to the tumour. Indeed the augmented DNA-content of human tumour cells can be sufficiently characteristic to serve as a valuable diagnostic criterion [Sandritter *et al.*, 1964; Sandritter, 1965; Böhm and Sandritter, 1975].

Benign human tumours behave like normal tissues in that they contain mostly 2c nuclei although some cells do occur in the S-phase.