CHROMOSOME TECHNIQUES

THEORY AND PRACTICE,
second edition

Chromosome Techniques

Theory and Practice

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Preface to Second Edition

The last eight years, since the first edition went to press, have witnessed tremendous advances made in all aspects of chromosome methodology. In addition to overall refinements to the methods already in vogue, outstanding progress has been achieved in the fields of human and mammalian chromosome methodology, including the study of malignant cells, mammalian chromosome analysis, and chromosomes in culture, together with somatic cell hybridisation, electron microscopy, high-resolution autoradiography and other modes of quantitation. These advancements in technology, along with a wide appreciation of the first edition—which resulted in its reprinting—added to the criticisms and suggestions received from reviewers, prompted us to venture on a new edition of the book. Each chapter has been rewritten with suitable additions, and deletions of the relatively less important techniques. Several new chapters have been incorporated but limitations imposed by the cost of production did not allow us to deal with the details of instrumentation on which a number of treatises are available.

In addition to those scientists, who had kindly given us the original microphotographs of their preparations for the first edition, the present edition has been further enriched by the inclusion of valuable photographs presented by the following experts, to whom we wish to express our sincere gratitude: Dr. E. G. Barry (Dept. of Botany, University of North Carolina, Chapel Hill); Prof. W. Beermann (Max-Planck-Institute Tübingen); Prof. J. G. Gall and Dr. M. L. Pardue (Dept. of Biology, Yale University, New Haven); Prof. H. Harris (William Dunn School of Pathology, University of Oxford); Dr. T. C. Hsu (M. D. Anderson Hospital, University of Texas); Prof. B. John (Dept. of Genetics, University of Southampton); Prof. B. A. Kihlman (Dept. of Genetics, Royal Agricultural College, Uppsala); Dr. T. N. Khoshoo (National Botanic Gardens, Lucknow); Dr. S. Ohno (Dept. of Biology, City of Hope Nat. Med. Center, Duarte, Calif.); Prof. S. P. Raychaudhuri (Dept. of Zoology, Banaras); Dr. M. Ray (Children's Hospital, Winnipeg); Dr. V. Sorsa (Dept. of Genetics, Univ. of Helsinki); Dr. M. S. Swamina-

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This endeavour would be considered a success if it proves useful to all workers interested in any aspect of chromosome study. Lastly, we are grateful to our publishers for their patience and meticulous care in attending to the revised manuscript.

Calcutta

A. K. Sharma
Archana Sharma

Preface to First Edition

The tremendous progress of the discipline of cytology within the last twenty years has been responsible for making the study of chromosomes a science in itself with its own theories and techniques and its own achievements. The continued enthusiasm for refinement in methods owes its impetus to the outstanding discoveries on the finer structure of chromosomes, the chromosomal basis of differentiation and the role and association of chromosomes in human abnormalities and cancer. Technological advances have also led to widening of the outlook on chromosome structure from a purely cytogenetical level towards a cytochemical and cytophysical analysis. This approach has given the cell biologist an insight into the pattern or organisation at the microscopical, submicroscopical, ultrastructural and even molecular levels. Achievements in methodology have further revealed the dynamic nature of chromosomes, in spite of their basic uniformity and their multiplicity in structure and chemistry, together with their development and physiology at different phases of growth.

A serious handicap to a cytologist is the absence of any comprehensive single treatise on the methodology of all aspects of chromosome structure and behaviour in all organisms and at different stages of their differentiation. Chromosome science, responsible for the unification in biology, should be viewed as a whole—as fundamental to all organisms and not as a series of compartmental sciences in separate realms of plants, animals or human beings. Not only does the need for a comprehensive treatise limit the study of chromosome science, but the absence of any book dealing with the chemistry of reactions between reagents and chromosomes is also a serious impediment to advanced research. The idea that the chemistry of the reactions is not known does not represent the true state of affairs. A number of reactions, though studied by experimenters, have not been published; those that are published, have not been compiled and presented for the convenience of the worker. The absence of any information on the chemical principles underlying chromosome techniques has resulted in the general practice of random trials with different fluids in search for a

suitable one.

This unscientific approach must necessarily be replaced by scientific and rational treatment. The difficulties that we faced as student, as research worker and as teacher, prompted one of us to start a probe into the technological aspects about twenty years ago. The intervening years have seen considerable advances in this field from different centres, including our own. The need for the presentation of the data obtained, in a critical and comprehensive form, is strongly felt—hence this publication.

In this book, efforts have been directed at presenting the technological aspects of chromosome study with particular emphasis on the principles underlying the different techniques and on the outlining of schedules. Methods have been described along with an account of their advantages, limitations and applicability, to enable the worker to plan a project directed either towards refining the methods given, discovering newer ones or applying them to various objects. As a natural sequence, the achievements too have been pointed out in brief. In order to meet the above ends a considerable amount of theoretical discussion has been incorporated which, it is hoped, would be of use to anyone interested in chromosome study.

This book is designed to meet the requirements of teachers, research workers and students alike, engaged in chromosome studies on plants, animals and human beings. No claim is made however to regard it as an encyclopaedia; for critical discussions on certain aspects of instrumentation, such as in electron microscopy or ultra-violet photometry, the reader is referred to the works of experts in the field. Efforts have been made to provide the widest possible fundamental knowledge for those whose only instruction in chromosome science would be this book, and maximum specialisation for those who intend to pursue it further. Modern developments in chromosome science have not only undergone evolution towards extreme specialisation but simultaneously towards greater simplification of the existing schedules. That is why chromosome science can now be pursued in any laboratory, however ill-equipped it may be, the category of research naturally depending on the availability of equipment. The object of the book will be achieved if it can meet the demands of workers engaged in this field in any type of laboratory.

The task has been made easier by the existence of certain books, invaluable in their own fields, chief of which are: *Histochemistry* by A. G. E. Pearse; *An Encyclopaedia of Microscopic Stains* by G. T. Gurr; *The Microtomists Vade-mecum* edited by B. Lee, and *Cytological Technique* by J. R. Baker. Our thanks are due to our co-workers in the field of cytology from several countries, whose good wishes gave us the impetus to finish this work. Special thanks are due to Prof. P. C. Koller of the Chester Beatty Research Institute, Drs. S. Makino and M. S. Sasaki of Hokkaido University, Drs. A. Levan and W. W. Nichols of South Jersey Medical Research Foundation, Prof. H. G. Callan of the University of St. Andrews, Drs. Wenner Schmid and T. C. Hsu of the University of Texas M. D. Anderson Hospital and Research Institute, Prof. J. H. Taylor of the University of Florida, Drs. H. H. Smith and W. Prensky of Brookhaven National Laboratory, Drs. P. Harris and D. Mazia of the University of California, Dr. J. Mitra of the University

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Contents

PART 1 PHYSICAL NATURE OF CHROMOSOMES

1 Introduction

The analysis of chromosome structure starts with the understanding of a clear delimitation between the chromosomes of lower organisms, including viruses and bacteria on the one hand and those of higher organisms on the other (Gay, 1966). In the former, the chromosome is a genophore, being essentially a long chain DNA molecule, not discernible under the light microscope and studied mainly through electron diffraction patterns and mutation and recombination data. In the latter, the chromosome is an extremely complex body, which has other constituents in addition to the DNA genophore, and it may be analysed under a light microscope. The genic effect on metabolism in the former can be immediately studied, whereas in the latter there is a wide gap and a series of reactions intervenes between the initial reaction at gene level and its manifestation in the phenotype. A concept of the evolutionary advance, step by step, from the genophore of the prokaryotes to the complex chromosome structure of eukaryotes, correlated with functional diversities and specialisation of segments, had been proposed (Sharma, 1969).

Research in the nineteenth and twentieth centuries has clearly established that 'chromosomes', which bear the hereditary materials or 'genes' in a linear sequence, are of permanent fibrous constitution. The term 'chromosome', meaning *colour body*, is based on its property to absorb certain dyes, thus showing basophilia. The importance and scope of the different techniques in the study of chromosomes can only be realised as the gradual advancement, aided through technical refinements resulting in the modern concept of chromosome structure, is outlined.

Notwithstanding the fact that the fibrous constitution of chromosomes has been unequivocally accepted, much discussion has been occasioned with regard to the longitudinal constitution of the thread, and the ultimate number of fibrous units composing it in Eucaryota. Leaving aside the *alveolar* theory, which has been completely discarded and in which chromosomes were supposed to be constituted of 'alveoles', reconciliation between the other two, i.e. the *chromomere* and *chromonema* hypotheses, has been

effected in later years. The filamentous nature of chromosomes was first visualised in the chromomere hypothesis. This hypothesis assumed that the chromosome is composed of longitudinally aligned chromatic granules, otherwise termed 'chromomeres', joined by an achromatic thread. The chromonema hypothesis, on the other hand, visualised a uniformly thick continuous structure in a chromosome, capable of spiralisation in mitosis.

The amalgamation of the two theories was brought about by Sharp (1943). He assumed that the varying distribution of the extra-chromatin substance, in addition to the one constituting the chromomeres, is responsible for the chromomeric and chromonemic appearance of the chromosome at different stages of the divisional cycle. In the later phases of division, when the deposition of extra-chromatin matter is heavy, the chromomeric appearance becomes pronounced. In telophase and earlier stages of division, the structure appears to be of nearly uniform thickness. It is now accepted by several authors that chromomeres represent specially differentiated portions of the chromonema thread, showing chemical reactions different from the interchromomeric segments. Whether the genes are located on the chromomeres or on the interchromomeric segments has also been debated. Chromomeres have also been assumed to represent tightly coiled regions of the chromosome thread (*see* Ris, 1957).

With regard to the multiplicity of the chromonemata, three different theories have been prosposed in the past, based on the number of threads constituting each chromonema. Darlington (1937, 1965) postulated the single-thread conception, in which the anaphase thread was supposed to be single, becoming double in the subsequent prophase. The double nature of the anaphase thread was maintained by Gates (1938), who also assumed that the chromosomes divided at the prometaphase condition, the metaphase thread being quadripartite in nature. The bipartite nature of the anaphase chromosome has been demonstrated by nitric acid vapour treatment. The other view held is that the anaphase chromosome is quadripartite, becoming 8-partite in the subsequent prometaphase (Nebel and Ruttle, 1936). Irrespective of the different theories held with regard to the unitary or multiple nature of the chromonemata, the generally accepted idea is that the anaphase thread is not unitary in nature. Opinions still differ as to the exact number of fibres present in the anaphase thread.

Later observations (Taylor, 1967), indicating the completion of DNA synthesis before zygotene have raised important issues regarding the initiation of meiotic pairing. The multistranded nature of chromosomes has been confirmed by various workers, both through ultrastructural and other evidence, and the implication of these findings has been discussed in several treatises and excellent reviews (Keyl, 1965; Sparvoli *et al.*, 1966; Gay, 1967; Sueoka *et al.*, 1967). The problem of interpreting the series of 200–250 Å thick fibrils in chromosomes has been outlined by Dupraw (1965), Wolfe (1965), Gall (1966) and Ris (1967).

The synchronous functioning of multi-stranded fibrils with respect to gene action has been explained in terms of a master gene, the rest being derogated to the category of slave genes (Callan, 1967; Whitehouse, 1967; *see* Edström, 1968).

With the aid of electron microscopy, ultrastructural units of chromosomes have been clarified. The minute lamellar constitution of the chromosomes has been claimed by a number of workers dealing with electron micrographs (Kaufmann and De, 1956; Ris, 1957; Kaufmann and colleagues, 1960), interpretation of the orientation of the molecules being different, however (Taylor, 1968). It has been suggested that 32, 64 or even 128 microfibrils may constitute the chromosome thread. This multilamellar concept has serious implications on the existing theories of crossing over and mutation as to how far all the lamellar units can undergo recombination of segments simultaneously, and how far mutation may originate in all the corresponding loci of a segment of chromosome lamellae. To explain mutation, therefore, fortuitous and simultaneous changes in the same locus in all fibrils have to be assumed. Recombination presents a similar problem where crossovers must involve all the fibrils at the same time (Uhl, 1965). To reconcile these discrepancies, the existence of a single long chain DNA molecule is often assumed but the gap between molecular and microscopic dimensions is too wide to be justified.

The theories of chromosome division do not necessarily require any modification in the light of the lamellar concept (Sharma and Sharma, 1958). When refinements in technique make it possible to reach the molecular level, more sub-divisions of lamellae may be clarified. The fundamental issue regarding their behaviour in anaphase is their segregation as binary units during division, and fortunately no case has yet been recorded where chromosomes have not behaved as binary units in anaphase. Therefore, on the basis of circumstantial evidence, a reasonable assumption is that, whatever may be the number of lamellae constituting the chromosome, the two daughter halves at anaphase are composed of an equal number of lamellar units. It may therefore be stated that the functional unit of the chromosome is the chromatid. Lamellar nature can be studied only with the aid of electron microscopy (*see* Chapter on Electron Microscopy), whereas the multiplicity of the chromonemata is revealed by acid or ammonia vapour (*see* Chapter 8). Such treatments not only expose the chromosome thread, but also the relational spiral between the two chromatids and the spirals between the constituent units of chromatids.

The refinements in technique have brought about a complete reorientation of outlook in the study of chromosome morphology. Formerly the conventional method of describing a chromosome was to refer to it as a *J* or *V* shaped structure, or rather as acro- or meta-centric, without any description of its morphology. This was principally due to the inadequacy of the techniques then available, which could not clarify the details of the structure. Gradually, as the need for an intensive research into chromosomal details was realised, techniques were invented from different centres (Flemming, La Cour, Lewitsky, etc.), which allowed further resolution of the details, including the clarification of primary and even secondary constriction regions responsible for the formation of the nucleolus. Further advances led to an understanding of the heterochromatic and euchromatic segments of chromosomes, which could be differentiated through temperature and other treatments. These two types of segments are characterised

by different staining cycles in the different phases of division. The presence of different types of heterochromatin, with regard to different functions, has been realised (Brown, 1966; Wolf and Wolf, 1969).

The molecular pattern has yet to be correlated with the observed transverse differentiation of chromosome segments, though several models have been outlined (*see* Hamilton, 1968). The centromere, secondary constriction regions, telomeric segments and other heterochromatic regions must fit in the molecular structure of the genophore, since they are regions which have been differentiated functionally. The occurrence of divalent cations, histones and even lipids are often regarded as transverse incorporations.

A major achievement in the study of chromosome science is the understanding of its dynamicity, replacing the concept of its uniformity and monotonous behaviour in all organs (Sharma, 1968, 1971). The dynamicity in chromosome behaviour in respect to differentiation and development has been seen clearly in *Drosophila* and other Diptera (Beermann, 1967; Pavan and Da Cunha, 1969). The synthesis of ribonucleoprotein for certain segments in the lampbrush chromosome at certain stages in vertebrate oöcytes as well as puffing at different segments of salivary chromosomes in different growth phases in *Drosophila* present direct evidence of chromosomal control of metabolism.

Further examples of this behaviour are the differential replications in meristematic and adult differentiated nuclei. While the behaviour of the chromosome thread follows the usual sequence in the former, it undergoes endomitotic replication in adult nuclei. This interpretation can explain the genic control of differentiation, which is maximum at the adult stage, as well as the apparently non-dividing state of the chromosomes. The cause of chromosome duplication, without separation, in adult nuclei has been attributed to regulated deficiency of the sugar component of DNA (Sharma and Mookerjea, 1954). The polytene state, due to endomitotic replication, has been confirmed by inducing division in these nuclei through IAA treatment by Huskins (1947) and by the precursors of nucleic acids and related chemicals in our laboratory. However, a generalisation from the evidence obtained from lower organisms implies that transcription and translation responsible for gene action and differentiation are not necessarily associated with gene duplication. The endomitotic replication, occurring with diploidy, and the polytenic constitution of some differentiated nuclei, are, however, observational facts, the association of which in differentiation requires clarification. These observations raise the problem of the transcribing limit of a DNA strand and the necessity of fresh strands for transcription. In any case, it is a clear index of the dynamicity of chromosome behaviour in response to the need for differentiation.

In the last several years considerable strides have been made in the study of chromosome structure, due to the invention of a number of pre-treatment chemicals. Special treatments, previous to fixation, have been responsible for a clear understanding of the structure of the different parts of chromosomes, including the centromere—the chromosome segment necessary for attachment and movement along the spindle. Knowledge regarding the

structure of the centromere was vague until its quadruple nature in metaphase was observed, which has been further clarified through later works. Refinements in methods have led to a clear understanding of the structure of the spindle and its relationship with the chromosome (Wada, 1966; Sakai, 1968).

Such changes in pattern, associated with phasic development, growth and differentiation of plants, indicate that, in spite of a basic genetic uniformity, the structural pattern of chromosomes is dynamic. Even the study of their chemical nature, discussed later, has shown that the chromosomes which are packaged for transmission are not identical in all respects with those of the somatic cells responsible for differentiation.

Further evidence of dynamicity and its control in the reproduction of species is seen in the chromosome behaviour in asexually reproducing species studied extensively in our laboratory. Chromosome complements of the somatic tissue universally exhibit numerical and structural changes and the constancy of the chromosomes is not maintained as such in different cells of the same tissue, but a chromosome *mosaic* is formed. This *regulated* abnormal behaviour is of great importance in species identification since the changed chromosome complements may enter into the growing apex of the vegetative shoots and form genotypically new individuals without the act of fertilisation. This outstanding example of dynamicity in chromosome behaviour, observed with the aid of refinements in techniques, shows the response to reproductive necessity under abnormal conditions.

The invention of methods for chromosome study in normal and cancerous cells of human beings has yielded further information of this dynamic behaviour suggesting that the chromosomes do not follow a normal pattern of behaviour in malignant cells. Mitotic instability, uncontrolled cell proliferation and mosaics of different chromosome numbers characterise the different tumourous and cancerous cells. The technique for such studies, though simple, was not available for a considerable period and its discovery has led to outstanding achievements in this field. The convenient schedules evolved for culturing leucocytes from blood have opened up new avenues of research and several congenital anomalies have been correlated with definite chromosomal characteristics. The advances in the study of human chromosomes have been outstanding. The significance of the dynamicity in chromosome behaviour, shown through recent developments in methodology, cannot be overrated since it has been instrumental in understanding the overall control of chromosomes in maintaining hereditary stability, as well in species replication and the hitherto unexplained processes of development and differentiation.

Finally, before dealing with the details of techniques, a few words about the modifications of chromosome structure, occasioned by physical and chemical treatments, is necessary.

Since the discovery of polyploidisation through colchicine, and mutation and chromosome breakage through x-rays, vigorous research on these and allied aspects is being carried out throughout the world. The fundamental and utilitarian implications of such findings will be outlined in their respective chapters. This aspect of study involves two lines of investigation,

one dealing with the techniques for inducing chromosomal abnormalities, and the other concerning the methods adopted for scoring the results.

For the study of the different aspects of the physical structure of chromosomes, varied techniques have been devised from time to time. The details of these techniques—their principles, applicability, drawbacks and recent developments—are discussed in the subsequent chapters.

REQUISITES

Chromosome study from living cells presents considerable difficulties. The phase difference or, more precisely, the difference in refractive indices between cellular components, being insignificant, it is difficult to resolve the components separately under the light microscope. For the study of chromosomes from living cells, ultrastructural pattern and quantitative estimation of chromosome components, different types of microscopy are employed.

In order to study chromosome structure under an ordinary light microscope, the tissue is killed and the tissue part selectively preserved without causing any appreciable distortion of nuclear matter, through a process known as 'fixation'. During this process, the cells are killed and dividing nuclei are fixed in the particular phase of division in which they were lying at the time of treatment. Chromosome study is usually performed after subsequent stages of washing, processing and staining in suitable dyes.

It has been observed that mere fixation alone may not prove adequate for the analysis of chromosome structure, especially of the somatic cells. This is particularly true for nuclei having a high chromosome number or very small chromosomes. In the former case, all the chromosomes form an aggregated lump, the individual structure thus not being discernible. In the latter case, on the other hand, the chromosomes may undergo too much swelling to allow any analysis of the details. The non-availability of any adequate method of fixation, and the lack of any suitable special schedule prior to fixation, led to the publication of a large number of papers in which the description of chromosome structure was either absent or inadequate. In the study of phylogeny or affinities between species, over-emphasis on the number of chromosomes with practically no reference to their structure, as is done in a number of cases, can also be attributed to these technical limitations. This is principally the reason why the role of structural changes of chromosomes in evolution was not properly assessed in the past.

The invention of a number of pre-treatment chemicals which are applied to fixation has considerably aided the study of chromosome structure in all its details. The principle of pre-treatment is mostly to bring about physical changes in the cytoplasm and nucleus which would help in clarifying the details of chromosome morphology. Thus, for the study of the physical nature of chromosomes, (*a*) pre-treatment, (*b*) fixation, (*c*) processing and (*d*) staining are the four essential steps which are discussed in the following chapters.

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