"Cytophotometric Studies on the Cell Nucleus"

(Ph.D. Thesis submitted by S.C.Frazer, December 1955)

SUMMARY

The work reported is an investigation of the amounts of deoxyribonucleic acid (DNA) contained in individual cell nuclei from a number of animal tissues, the amounts being estimated by microspectrophotometric methods.

PART I outlines the history of microscopy, and in particular the application of the quartz microscope, in the ultraviolet region of the spectrum, to biological specimens. Historical aspects of histochemistry and cytochemistry, and of quantitative microspectrophotometric procedures, are also described, together with a brief review of the development of current knowledge of the nucleic acids.

PART II consists of a critical survey of methods which have been used for the estimation of deoxyribonucleic acid in single nuclei, and describes a photometric procedure for this purpose, based on density measurements on photomicrographic negatives. The procedure has been developed for use in either the visible or the ultraviolet regions of the spectrum, and its application to the estimation of DNA by three distinct techniques is described. These methods are ; ultraviolet absorption measurements on unstained isolated nuclei; visible light absorption measurements on Feulgen-stained nuclei; and visible light absorption measurements on nuclei following staining with methyl green.

PART III presents the results of a series of comparative measurements of the DNA contents of single nuclei from a number of normal and abnormal tissues in the adult rat, and a small series of measurements on embryonic chick tissues. A statistical assessment

assessment/ of the significance of the differences between nuclei has been made.

PART IV is a discussion of the results obtained, and of their significance in relation to the possible role of DNA as a chemical basis for the concept of the gene. It is concluded that in the rat the amount of DNA contained in diploid somatic nuclei is nearly constant, irrespective of the tissue of origin, although small differences between nuclei occur. In those rat tissues which have a proportion of tetraploid or octoploid nuclei, there are found nuclei having twice or four times the usual amount of DNA. Spermatozoa have approximately half that amount. The results are in keeping with the postulated role of DNA in the transmission of hereditary characters.

Publications included : Experimental Cell Research, Vol 4, p.316 (1953)
and Experimental Cell Research, Vol 6, p.367 (1954)

------0------

Cytophotometric Studies on the Cell Nucleus

by

Samuel Cherrie Frazer.

Thesis presented for the
Degree of Doctor of Philosophy
in the
University of Glasgow, Scotland.

December, 1955.

"Instrumental or mechanical science is the noblest and above all others the most useful."

Leonardo da Vinci (1500)

"For now we see through a glass, darkly ....."

First Epistle of St. Paul to the Corinthians.

"The many potential sources of error in individual measurements may explain a large part of the variance within a cell population, but they are not such as to invalidate the whole photometric approach in cytology."

A.W. Pollister (1952)

"A good training in histology is one of the worse backgrounds possible for cytochemical work."

J.F. Danielli (1953).

Acknowledgments

I should like to record my very sincere gratitude to Professor J.N. Davidson for his constant guidance and encouragement while this work was in progress; an ultra-violet microscope (the property of the Medical Research Council) was made available to me through the courtesy of Professor Davidson.

I am indebted to Dr. S.T.R.S. Mitchell, Lecturer in Chemistry in this University, for allowing me the use of a densitometer, and for helpful discussions on spectrographic techniques; to Dr. R.A. Robb, Lecturer in Statistics in this University, for help and advice on the statistical treatment of some of the measurements; to Mr. A.M. Andrew, B.Sc., for advice in connection with voltage stabiliser circuits; to Dr. N.B. Kurnick, who kindly supplied me with a reference sample of methyl green dye; to all those colleagues with whom I discussed various aspects of this work, and especially to Dr. R.Y. Thomson, who collaborated with me in a number of the experiments, as detailed in the Note overleaf; to Dr. I. Leslie, who provided chick heart fibroblast tissue cultures.

The work was carried out while holding a teaching appointment in Biochemistry in the University of Glasgow,

and the laboratory facilities used were those of the Department of Biochemistry in that University.

Notes

1. Certain of the experiments recorded in this thesis were carried out jointly with Dr. R.Y. Thomson, namely, those on Feulgen-stained nuclei of which the results are presented in Figures 8,11,12,13,14, 15 and 17. In these experiments, the isolation of nuclei and the measurements on the photographic negatives were carried out by Dr. Thomson, while the staining, mounting and photomicrographic procedures were performed by the author. The statistical calculations were carried out jointly.

2. The experimental work was performed between December 1950 and March 1953; the bibliography includes references to relevant publications up till November 1955.

3. The major portion of the work reported in this thesis has been published, as follows:-

   a) "Photometric Estimations of Deoxyribonucleic Acid in Individual Cell Nuclei",
   by S.C.Frazer and J.N.Davidson,
   Experimental Cell Research, Vol.4, p.316 (1953).

   b) "The Deoxyribonucleic Acid Content of Individual Rat Cell Nuclei",
   by R.Y.Thomson and S.C.Frazer,
   Experimental Cell Research, Vol.6, p.367 (1954).

Copies of these publications are appended.

CONTENTS

Contents (continued)

Contents (continued)

Contents (continued)

PART I.

Introduction.

1.1. Historical survey on microscopy and the cell theory.

Following on the invention of the compound microscope by Galileo about 1609 (and by the brothers Janssen in Holland about the same time), little use seems to have been made of the instrument as a tool in the study of plants or animals until the middle of the 17th century, when Malpighi in 1661 made some observations on capillary blood vessels. The first detailed description of the microstructure of plants, fungi and insects, however, was made by Robert Hooke in his Micrographia, published in 1665. The drawings which illustrate that work are remarkable for the detail shown, and it is probable that the instrument which he used, though suffering severely from optical aberrations and from the necessarily restricted aperture of the objective lens, had nevertheless a useful magnification of about 100 diameters. In suitable plant tissues he was readily able to distinguish the outlines of the cells - indeed it is to Hooke that we owe the word "cell" - and some of the illustrations may also show indications of the presence of structures within the cell. Hooke used the term "cell" in the sense of a small compartment with defined walls - he also named them "pores" - but he appears to have had little inkling of the functional importance of the small units which he saw. It is

scarcely surprising that the much smaller cells of animals were not revealed by his experiments.

Later in the 17th century, Hooke's observations were repeated and extended by Malpighi and Grew, who called the microstructures "utriculi" and "bladders" respectively. Simultaneously in Holland, Swammerdam and van Leeuwenhoek were independently pursuing microscopical studies of a similar character, though mainly directed towards improving the crude early instruments.

Following the death of van Leeuwenhoek in 1723, few, if any, significant observations were recorded on the microscopic structure of living organisms for nearly a century. The first half of the nineteenth century, however, saw a great increase in interest in the microscopic anatomy of plants, although most botanists at that time were greatly concerned with taxonomic problems, and plant physiology as a science was almost non-existent. In 1831, however, the botanist Brown drew attention to the occurrence within the plant cell of an apparently denser body, or kernel, suspended in a fluid juice. A few years later, Schleiden, distinguishing between nucleus and cytoplasm, enunciated the elements of modern cell theory, as applied to plants, while the histologist Schwann, from studies on animal tissues, declared that plant and animal cells had many features in common, including, of course, the readily recognisable

nucleus.

The middle years of the nineteenth century saw extensive advances in knowledge of the microscopic anatomy of both plants and animals. The functions of the nucleus, however, remained undecided until about 1880, when several workers, and especially Schneider and Strasburger, recorded in detail the stages of the mitotic cycle as an essential part of the process of cell division. There remained the correlation, early in the present century, of the known facts of Mendelian inheritance with the observed behaviour of chromosomes during reduction divisions and during fusion of the gametes, and the main physical outlines of present-day cell theory had been drawn.

1. 2. Historical development of Histochemistry and Cytochemistry.

The science of histochemistry began almost as soon as histologists began to speculate on the meaning of the structures which their microscopes revealed, and, as in the early days of histology, the foundations of the science were largely laid by botanists. Thus in 1826 we find Caventou describing the use of the iodine reaction for starch in microscope preparations (a test first used on a macro-scale, some ten years earlier, by Colin). Raspail, in 1829, applied the xanthroproteic reaction for proteins

to plant sections, and also studied the fertilisation of grasses, using the starch-iodine reaction as a histochemical tool. These and other investigations were published as early as 1830 in his "Essai de Chimie Microscopique Appliquée à la Physiologie".

Further advances in microscopical chemistry, however, were dependent on the development of suitable specific chemical tests for many constituents of plant and animal cells, that is to say, on progress in this branch of general biochemistry. In many cases, a lag period of thirty years or more occurred between the introduction of a chemical test and its application to the microscope specimen. Thus in 1844 Millon described his mercury reaction for proteins, but it was not until 1888 that Leitgeb applied essentially the same test to reveal the location of proteins in tissues. Similarly Klebs in 1868 demonstrated the peroxidase activity of pus by its action on guaiac, while in 1900 Brandenburg showed that this enzymic reaction could be made the basis of a selective staining method for the granules of leucocytes.

Progress in histochemistry became very slow during the latter half of the nineteenth century, largely owing to the rapid widening in the scope of orthodox histology, brought about by the introduction of the aniline dyes. A great deal of effort was directed to the study of the staining

reactions of tissue sections, and to the minutiae of morphology thus revealed, although little attention was paid to the question of why particular structures could selectively bind particular dyes. Many chemists felt that staining by dyes was essentially a physical process, a view strongly put forward by A. Fischer (1898), who considered that the only basis for selective staining lay in differences in the adsorptive forces between the dyes and the cell structures with which they became linked. A few workers, however, maintained the view that differences in staining properties must imply underlying chemical differences. Thus in 1873 Miescher reported on the selective affinity of nuclear chromatin for the basic dye methyl green, while Ehrlich (1878) showed that heat could increase the affinity of proteins for azo dyes, a process which we now know to be due to the liberation of reactive groups in the protein molecule by heat denaturation. In general, little was known of the nature of the dye complexes formed in the stained sections, beyond vague speculations on the basic or acidic nature of cell structures as shown by their dye affinities. However, Mann in 1902 clearly put forward the dual role of staining as, firstly, an aid to the study of morphology, and secondly, as a means of revealing the localisation of cell constituents, by means of their chemical reactivity.

It is not, of course, necessary that the chemistry of a staining process should be understood in detail before conclusions can be drawn from its results - indeed at the present time many staining techniques remain on almost the same empirical basis as when they were first introduced, despite extensive chemical studies to ascertain their mechanisms. Worthy of mention in this respect is the Weigert (1884) technique for histological demonstration of degeneration in myelinated nerve fibres, whose application has contributed so much to our knowledge of the interconnections of the central nervous system. Yet the precise nature of the degeneration of the myelin sheath which accompanies nerve section is only now being fully investigated.

Methods involving the application of enzymes to tissue sections are of interest both to the biochemist and to the histologist, but are of comparatively recent origin. The early use of enzymes, mainly proteolytic, in the period 1870-1895, was intended to facilitate the isolation and study of particular structures rather than to reveal the chemical nature of the material on which the enzymes were made to act. Thus Miescher in 1871 used pepsin digestion of cytoplasm as an aid to the isolation of nuclei, while in 1875 Stirling similarly removed cytoplasmic material by the aid of pepsin, when isolating elastic fibres. By contrast,

present-day use of highly specific and highly purified enzymes in histochemistry is intended to aid in the localisation of cell components, frequently by means of less specific staining techniques applied before and after the enzyme treatment.

A paper of fundamental importance in histochemistry appeared in 1899, when Hardy showed that the final picture given by any stain or combination of stains could be profoundly altered by the technique of preparation of the specimen, and was not solely related to the chemical nature of the material visualised. Thus the method and duration of exposure to the fixative, the chemical nature of the fixative, the details of staining and of differentiation, even, in some cases, the nature of the mounting medium, all influence the final appearance of the specimen, and the interpretation of any microscopic picture, in terms of either morphology or chemistry, can be dangerously in error unless all possible regard is paid to the effects of the processes used in the preparation of the specimen.

One effect of the increasing awareness of the occurrence of fixation artefacts was to direct attention away from fixed and stained preparations, and towards the study of the living cell. Since no methods of chemical investigation existed which did not involve some degree of damage to the specimen, the studies of experimental

cytologists in the period 1900-1935 were largely in the field of dynamic morphology, such features as contractile vacuoles, ciliary movement, and mitotic figures being extensively studied from the physical aspect. The results of these studies proved singularly difficult to interpret unless considerable assumptions were made regarding the physicochemical nature of the motile structures studied, and since reliable information on chemical and enzymatic loci within the cell was decidedly scanty, a number of cytologists again turned their attention to studies of a histochemical type.

Although this period was not, in general, a fruitful one as regards chemical studies made with the aid of the microscope, it saw the introduction of one cytochemical technique of outstanding value, by Feulgen and Rossenbeck in 1924. The Feulgen reaction, as it is commonly known, is now regarded by many workers as revealing the location and amount of deoxyribonucleic acid within the nucleus, while leaving the cytoplasm unstained. (This reaction will be considered in some detail in a later section.)

The period from 1935 to the present time has seen a considerable revival of interest in the application of methods of biochemical analysis to tissue sections and to single cells. Among the pioneers of modern histochemistry, special mention must be made of the work of Lison, whose textbook,

"Histochimie Animale", first published in 1936, emphasises the need for methods which retain the specimen in a condition close to that of the intact cell or tissue, while still allowing valid chemical information to be obtained. A very great advance towards this goal was made by Gersh, in 1932, when he described a technique for dehydrating and embedding portions of tissue without exposing the cells to the action of any chemical fixative. This technique of freeze-drying was a development of the work of Altmann in 1890, and since it did not involve any drastic alteration in the chemical nature of cell constituents, but only in their physical state, it has proved of especial value in the study of cell proteins, notably the enzymes.

In the past twenty-five years, a very large number of reactions have been described as suitable for application to tissue sections, and histochemical tests now exist for the detection of most of the major groups of biological compounds, and for many inorganic constituents of the cell. The great majority of these reactions are as yet purely qualitative in character, and in many cases there are grounds for suspicion that their specificity and their accuracy of localisation may leave much to be desired.

The minimum requirements for a satisfactory histochemical technique have been summarised by Glick (1949) as follows:-

1) The preparation of microtome sections in which there has been no significant alteration in the position of the constituent being investigated.

2) A reagent which is specific for this tissue constituent.

3) A reaction between the reagent and constituent which is of such a nature, and rapid enough, to obviate diffusion of the constituent or of the reaction product.

4) A reaction product, this trapped in situ, which is capable of being visualised.

As Glick somewhat sorrowfully observes, the frequency with which these minimum requirements can be met is still very low.

Since the publication of Lison's textook in 1936, many workers have made valuable contributions to the study of cellular chemistry by microscope methods; outstanding names include those of Gomori, Linderstrom-Lang, Holter, and Takamatsu, while accepted methods have been gathered together in textbook form by Glick (1949) and by Pearse (1953). A second edition of Lison's textbook also appeared in 1953.

1.3. Ultraviolet microscopy and microspectrophotometry.

Towards the end of the nineteenth century, spectroscopists were in possession of basic information on the properties of light in the near and middle ultraviolet (U-V) regions of the spectrum. It was known that glass of all

types then available was impenetrable to light of wavelengths shorter than about 3100 A, whereas quartz and a few other crystalline minerals were transparent to ultraviolet light in the wavelengths down to about 2200 A. Since several of the U-V-transmitting minerals are also doubly-refracting, their use for most optical components is impracticable, and accordingly quartz is commonly used for lenses and prisms required to transmit U-V light.

The goal of a microscope operating in the ultraviolet region was attractive to optical designers, since it was known that the theoretical resolving power of a microscope is given by the formula

$$d = \frac{1.2\lambda}{NA(Cond) + NA\ (obj)}$$

where d is the minimum separation between two points which can be resolved,
λ is the wavelength of light used,
NA(cond) is the numerical aperture of the condenser,
NA(obj) is the numerical aperture of the objective.

If the mean wavelength of white light is taken as 5600 A, a microscope operating in ultraviolet light at 2800 A, having comparable lenses to the normal visible light instrument, will possess resolving power improved by a factor of two.

By about 1900, it was clear that high power lenses designed for visible light work had almost reached the practical limit of numerical aperture, and that therefore

increased resolving power could only be obtained by the use of shorter wavelengths, outside the visible spectrum.

The first practical design of an optical system for U-V microscopy appeared in 1904, when Kohler, working with von Rohr, described an instrument based on normal principles of visible light microscopy, but having the optical system made of quartz in lieu of glass. The lack of alternative refracting media to unite with quartz to make achromatic U-V combinations led these workers to the conclusion that it was necessary for the objectives to be monochromats, i.e., designed to operate satisfactorily at one wavelength only, and they therefore designed and produced a series of high power fused quartz lenses suited to high-resolution microscopy at chosen wavelengths in the middle ultraviolet region. The excellence of their optical design may be judged from the fact that nearly forty years elapsed before they were appreciably improved.

Although the Kohler and von Rohr objectives were computed for strictly monochromatic light of specified wavelengths, it has since been shown by Cole and Brackett (1940) that they can be successfully used over a wide range of ultraviolet wavelengths, provided the focus is adjusted, and provided the radiation is very nearly monochromatic. The theoretical tolerable bandwidth at 2800 A was calculated by Cole and Brackett to be about 5A, while Johnson (1929)

had shown that in practice even a bandwidth of 12A would produce an unsatisfactory image. This serious limitation, although not detracting from the excellent performance of which lenses of this series are capable, had a lasting influence on the design of equipment, since no monochromator could be expected to provide even low-intensity U-V light of so narrow a bandwidth. Attention was therefore concentrated on sources of U-V radiation which were in themselves monochromatic, e.g., sparks between suitable metal electrodes, and gas discharge tubes, especially mercury arcs. Relatively simple monochromators, using quartz prisms, are able to isolate single spectral lines from such sources, with good intensity and high purity. The principal disadvantage of systems of this type lies in the limited number of wavelengths at which observations can be made, as well as the tedious refocussing required each time a change of wavelength is desired.

Focussing problems have been regularly encountered since the earliest days of ultraviolet microscopy, the problem of securing accurate focus being rendered more acute by the diminished depth of focus which accompanies the use of short wavelengths of light; with the highest power U-V objectives, an error of 0.5 μ in the focus setting produces a noticeable deterioration in the image. A number of means have been adopted to simplify focussing, none being entirely successful.

The Kohler instrument was fitted with a fluorescent searcher, an auxiliary quartz ocular containing a fluorescent screen and glass magnifier. Since the image on the fluorescent screen was at low magnification, it was reasonably bright, but its very smallness rendered critical focussing impossible except by trial-and-error exposures on photographic plates. Most workers have attempted direct focussing in the image plane, using transparent screens of fluorescent material in lieu of the ground glass screen used in visible light; in general, these attempts have been unsuccessful, except at low magnifications, since the available intensity of monochromatic ultraviolet light has been too low, after passing through the optical path of the microscope, to give more than a detectable glow on the screen. One exception has been the instrument described by Lavin (1943) in which the light source is a spiral low-pressure mercury resonance lamp, giving high intensity radiation at 2536 A. Using an activated willemite screen, Lavin has reported that focussing at full magnification is possible provided the observer is fully dark adapted.

Since the alteration in focus on changing from a visible-light system to quartz optics should be constant, it has been suggested (Swingle and Briggs, 1907) that it should be possible to focus in visible light at a particular wavelength, and thereafter change the focus by a predetermined

amount when moving to the U-V region. In practice, it is exceedingly difficult to construct a fine-focussing system whose backlash will be less than the tolerable error in focussing, and even minor changes in ambient temperature may be enough to destroy the reproducibility of the focus setting. Since the ultimate criterion of successful focussing is the production of an acceptably sharp photographic negative, most workers have found that it is best to find the approximate focus by fluorescent searcher, and thereafter a series of photographic exposures at closely adjacent focus settings usually yields a satisfactory picture.

The primary cause of difficulty in focussing, of course, is the monochromatic nature of the objectives designed by Kohler and von Rohr, and truly achromatic objectives to cover both visible and U-V regions would effectively solve the problem. Partially achromatic lenses have been devised, based on combinations of quartz and fluorite, but they are semi-achromatic only over a limited range in the ultraviolet, and not in the visible region. They do not, therefore, represent a great advance over all-quartz lenses, except possibly for microspectrophotometric work over a limited range of wavelengths. Much greater success has been achieved in the design of lenses based not on refraction by quartz, but on reflection at curved surfaces. Suitable

combinations of reflecting surfaces have been computed by Brumberg (1939, 1943) and by Burch (1947), the resulting lenses being achromatic through the range 2000-7000 A. They suffer from the serious drawback that the numerical aperture cannot exceed 0.65 (approximately), since one mirror must inevitably partially obscure the other, and also since such objectives are not by themselves suitable for liquid-immersion. The latter drawback has been overcome by Burch by means of an additional immersion-type lens between the mirrors and the object, giving an over-all N.A. of 0.98.

Combinations of reflecting surfaces and refracting elements have been used by Lee and Gray to give reasonably good achromatism down to about 2200 A, with a N.A. of 1.0, but lenses of this type have not yet come into general use. Wilkins and Norris (1952) have described a similar achromatic lens. It seems likely that further developments in reflecting-refracting objectives will greatly simplify the work of the ultraviolet microscopist, at least as far as focus-finding is concerned.

Suitable light sources for U-V work have also been a source of difficulty, since the ideal properties of such a source are to some extent mutually incompatible. The microscopist would wish for

1) high specific intensity (ideally, a point source),

2) numerous closely adjacent spectral lines in the desired wavelength range, or alternatively, a uniformly bright continuum throughout that range,

3) stability over periods of perhaps 2-3 hours,

4) minimum interference with adjacent electronic equipment,

5) compactness, for ease of mounting in the optical path,

6) moderate cost.

These requirements can be met in the visible region by ribbon filament tungsten lamps, or by point sources such as the "Pointolite", an arc struck between tungsten balls, one of which, when incandescent, is used as the light source. Arc or spark sources as commonly used for ultraviolet work, however, have serious drawbacks, and particularly if it is intended to use the microscope in association with photo-electric measuring equipment, since the radiated interference from an open arc or spark requires extensive screening for its elimination. Open arc or spark sources are also notoriously unstable, even where rotating electrodes are used, and where photometric work is being undertaken some form of compensating device must be employed, e.g., a second photocell whose function is to record, and compensate for, the fluctuations in the light source. Spark sources have been extensively used by Caspersson (1940a) and were employed in the original Kohler instrument (1904).

Low pressure arcs in mercury vapour, in a quartz tube, can readily provide almost monochromatic radiation at 2536 A (the so-called mercury resonance line). Other light from the arc can be removed by a single-prism monochromator, or by liquid filters, as described by Bowen (1946). As mentioned earlier, Lavin (1943) based his instrument on a lamp of this type, but, in general, the restriction to one wavelength, together with the low specific intensity of the source, limit the usefulness of the mercury resonance lamp.

High pressure mercury-vapour arcs were used by Caspersson (1940a) in conjunction with a single-prism quartz monochromator, their stability being improved by water cooling, and by stabilising the power supplies. Even with these precautions, it was found necessary to use a double photocell photometric system to compensate for lamp fluctuations, when quantitative photometry was undertaken. Mercury arcs, however, can provide a number of useful lines in the region 2300A - 3300 A, the relative intensities of the lines being dependent, to some extent, on the pressure of the mercury vapour.

For the satisfactory recording of absorption spectra in the ultraviolet region, it is desirable to use a source giving a continuous spectrum, from which the desired wavelengths can be isolated in turn by a suitable monochromator.

The small hydrogen arcs employed in present-day quartz spectroscopes and spectrophotometers are of insufficient intensity for microscope work, but partial success with higher powered hydrogen arcs has been reported (Scott and Sinsheimer, 1950). At the present time no single light source is ideal for all purposes, and the type selected would therefore depend on the nature of the investigation.

In his early studies with the U-V microscope, Kohler reported that he observed greatly increased contrast in photographs of unstained biological specimens, as compared with visible light photographs of the same objects. Two main reasons can be adduced to account for this effect; firstly the refractive index differences between the specimen and its mounting medium are accentuated at shorter light wavelengths, and lead to an apparent increase in contrast; secondly, in plant and animal tissues, many compounds specifically and intensely absorb light at particular wavelengths, and therefore their distribution within the specimen will become apparent in the form of less dense areas on the photographic negative.

The improved resolution obtainable in the U-V instrument was utilised by Barnard and Elford (1931), by Lucas and Stark (1931), by Wyckoff, Ebeling and Louw (1932), and by Manton and Smiles (1943) in connection with morphological studies on viruses and on chromosomes, but, in general, the

inconvenience of the quartz instrument, coupled with its high cost, have outweighed its advantages for high-resolution observation. In recent years, the advent of electron microscopy has provided increases in magnification which completely overshadow the small gain obtainable by working in the U-V region.

It therefore seemed not unlikely, by about 1935, that ultraviolet microscopy might be relegated to the status of a scientific curiosity. This state of affairs was radically altered, however, by the work of Caspersson (1936, 1940a,b, 1950a,b) who showed that well-established principles of absorption spectroscopy can be applied, on a micro-scale, to such unpromising material as the histologist's tissue sections. The essentials of the technique are simple; the specimen under study, e.g., a portion of a plant or animal cell, is illuminated in monochromatic U-V light in a quartz microscope. Measurements of the U-V light intensity in the image plane of the microscope are made, either electrically or photographically, for the portion of the image corresponding to the part of the specimen under study, and for an adjacent clear area. Comparison of these light intensities allows calculation of the proportion of the incident light which has been absorbed by the specimen. If the wavelength of light used is then changed, and similar measurements made over a suitable range of wavelengths, then an absorption

spectrum curve can be drawn for the portion of the cell under study. Admittedly the absorption spectrum obtained is a composite one, derived from all the molecules through which the radiation has passed, but as will be seen later, only a few cell constituents have sufficiently high specific extinctions, or are present in sufficient concentrations, to exert any marked influence on the cell's absorption of ultraviolet light.

It will be noted that the ultraviolet microspectrophotometer differs considerably from the spark spectrograph, in that for microphotometric purposes the specimen is irradiated by light of one wavelength only, at one time, whereas in the macro-instrument the sample is usually placed between the light source and the entrance slit of the spectrograph, and receives, simultaneously, light of all wavelengths present. The microspectrophotometer corresponds more closely to present day spectrophotometers in which the specimen is exposed, in turn, to light of each of a range of wavelengths covering the spectral region concerned, and separate measurements of optical density are made at each wavelength.

In orthodox absorption spectroscopy, the sample usually consists of a solution or liquid, contained in a cuvette whose dimensions are known, and failure to dissolve the sample completely will invalidate the method. Tissue sections, or

whole cells, on the other hand, can usually be regarded as containing materials in precipitated solid form, or else as colloidal gels which may be far from being optically homogeneous. Further, the precise sample thickness may be unknown, or varying continuously, as in a living cell, so that the precision attainable in normal spectroscopy cannot be automatically obtained in microspectrophotometry. Caspersson (1950), however, has pointed out that these considerations need not prevent a series of measurements being made on the same structure at different wavelengths, so that the absorption spectrum of the structure may be recorded with reasonable reliability. Since several cell constituents show typical absorption curves in the U-V region, a qualitative analysis of a structure may be possible, at least as far as U-V absorbing constituents are concerned. Where quantitative observations are to be made, however, serious optical problems arise. For example, the falling off of light intensity as it passes through a colloidal system is only partly due to specific absorption by molecules of that system. A considerable proportion of the incident light is lost by scattering, and cannot be ignored in microspectroscopy of objects such as cells. Further, the sizes of structures which one might wish to measure within the cell may be approaching the limit of microscope resolution, and therefore not greatly larger than the wave-

length of light used. In these special circumstances, the normal laws of light absorption might well be seriously in error.

This specific problem has been investigated in considerable detail by Caspersson (1936, 1940a, 1947, 1950b), and only his conclusions can be mentioned here. He considers that it is theoretically possible and technically feasible to construct valid absorption curves for structures whose size is not less than three times the wavelength of the light used, and provided the necessary optical conditions are fulfilled in the instrument and in the object, it is permissible to calculate, from analysis of the absorption curves, the concentrations of selectively absorbing substances, by application of the normal Beer-Lambert laws of light absorption by homogeneous systems. According to Caspersson, the essential requirements in the instrument are:-

1. It must be possible to determine the extinction of the specimen, with an accuracy of 1% or better, otherwise valid analysis of the absorption curves will not be possible. Extinction measurements must be possible over a large number of closely adjacent wavelengths.

2. The optics must fulfil Abbe's sine law, and the objective must have a N.A. of not less than 0.85 for large objects, or 1.25 for the smallest objects. If these conditions are met, then errors due to light scattering can be corrected for.

3. Optical conditions in the specimen must be accurately known, especially as regards refractive index of specimen and mounting medium. The medium should permit some swelling of the colloidal particles of which the specimen is composed.

In order to fulfil these instrument requirements, Caspersson and his collaborators have constructed comprehensive arrays of equipment, with which, it is claimed, microspectrophotometric measurements can be carried out on amounts of material of the order of 10^{-12} mg. The apparatus, in addition to the quartz microscope and elaborate photoelectric measuring equipment, contains devices for measuring the direction and intensity of light scattering around microscopic objects, and for direct measurement of the thickness of microscope specimens. The equipment in current use has been described by Caspersson (1954, 1955). It is sufficiently elaborate and costly to place it beyond the reach of most research institutions.

It is not always necessary, as Caspersson himself has pointed out (1950b), to be able to assess the magnitude of every error, or to eliminate all unknown factors in an experiment, so long as absolute measurements are not desired. For example, one might wish to compare the concentrations of a component in two similar cells which have been subjected to the same preliminary treatment. Since the instrument errors will, in general, be the same in each

case, it may be permissible to neglect them altogether, provided the magnitude of the effect to be measured is considerably greater than any error due to poor reproducibility of the measurements.

The outlines of a microspectrophotometer of the Caspersson type are given in Figure 1.

Caspersson's views on the validity of U-V spectrophotometry as applied to minute specimens such as portions of animal cells have not passed unchallenged. Commoner (1949, 1950) and Commoner and Lipkin (1949) have raised theoretical objections, especially as regards the validity of the assumption that semi-solid materials behave similarly to solutions, as regards the quantitative aspects of their light absorption, and have suggested that nucleic acids in particular may show anomalous apparent extinctions, owing to the elongated shape of the nucleic acid molecule. This effect, they claim, is likely to introduce serious errors if the molecules are not randomly orientated. Such orientation effects are unlikely to be seen in dilute solutions, but are not improbable in precipitated nucleoproteins such as are present in microscope specimens. In support of this contention, they cite the work of Schmidt (1937) who found evidence of orientation within chromosomes, and they also consider that Astbury's (1947) "stack of coins" model for the molecular structure of deoxyribonucleic acid (DNA)

Figure 1.

General arrangement of a microspectrophotometer as used by Caspersson (from Caspersson, 1950).

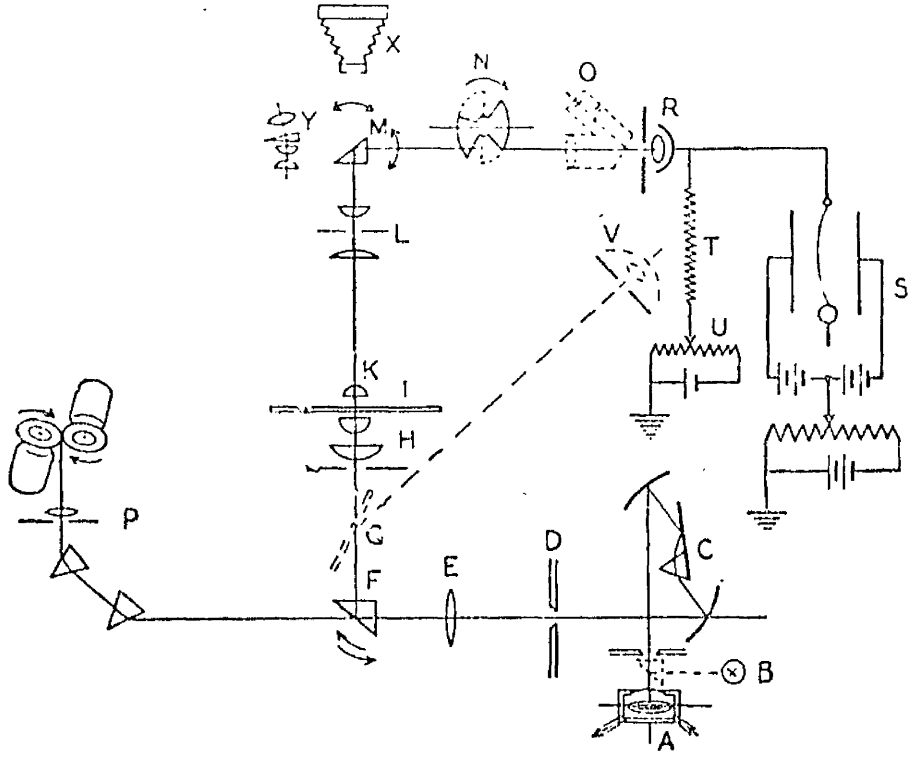

Figure 1.—Apparatus used by Caspersson for taking absorption measurements in ultraviolet light in microscopic sections. *A*, mercury lamp. *B*, tungsten band lamp. *C*, Monochromator. *D*, second monochromator-slit. *E*, lens. *F*, movable 90° quartz prism. *G*, quartz plate (used with photocell *V* to compensate for changes in the lamp). *H*, condenser. *I*, object. *K*, objective. *L*, ocular with adjustable diaphragm. *M*, accurately movable prism of fused quartz. *N*, rotating sector. *O*, telescope for centring. *P*, Köhler's rotating spark gap arrangement. *R*, photocell. *S*, electrometer. *T*, leakage resistance. *U*, four-step potentiometer. *X*, camera. *Y*, Köhler-focuser, for the ultraviolet, interchangeable with prism *M*. From Caspersson (1950)

provides supporting evidence for their views. Caspersson (1940b), however, failed to confirm Schmidt's findings on chromosomes, while the Astbury model for the DNA molecule is not in accord with recent observations (Watson and Crick, 1953). Seeds (1953) has reviewed the relationships between crystal form and ultraviolet absorptions.

Perhaps more significance should be attached to the second criticism of Commoner and Lipkin, namely that the calculation of the absolute amount of nucleic acid in a structure by microspectrophotometry is only valid if it can be shown that the light absorption is strictly related to the number of light-absorbing molecules present, i.e., that the substance being measured obeys the Beer-Lambert laws under the conditions of the experiment. Caspersson's corrections for anomalous absorption and light scattering are not in themselves sufficient to counter this criticism, but it is difficult to visualise any test objects which might be used to establish, rigorously, the validity of the Beer-Lambert laws of light absorption in this particular case. Comparative measurements on similar structures, however, may not be greatly in error, if the optical conditions in both objects are the same.

Caspersson and his co-workers have extensively employed U-V microspectroscopy in the study of the relationship between protein accumulation and nucleic acid

concentrations, both in the cell nucleus and the cytoplasm. A large number of relevant publications are listed by Caspersson (1950b), but it is perhaps worth while to summarise here certain findings which are especially relevant to the present study.

1: Ultraviolet microscopy does not of itself distinguish between absorption due to RNA and that due to DNA, nor does it distinguish between nucleic acids and free nucleotides, nucleosides, etc.

2: The ultraviolet absorption spectrum of a chromosome at metaphase is similar to that of a solution of nucleic acid in vitro while in Drosophila salivary chromosomes the so-called "dark bands" have also a spectrum typical of nucleic acid. (Caspersson, 1936, 1939, 1940b; Caspersson and Schultz, 1938).

3: The nucleolus, as judged by its U-V absorption spectrum, also contains nucleic acid. (Caspersson and Schultz, 1940: Caspersson, 1941). On the evidence of non-stainability with Feulgen reagent, this nucleic acid is presumed to be RNA.

4: In addition to the main peak at 2600A characteristic of nucleic acid purines and pyrimidines, the absorption spectra of chromosomes and nucleoli have a shoulder at 2750 A, corresponding to the presence of proteins containing cyclic amino acids. (Caspersson, 1941, 1947, 1950b).

5: "In the reproduction of the linearly arranged gene-chain (chromosome), the nucleic acids (participating in the processes for cellular protein synthesis) are of the ribodesose (i.e. deoxyribose) type". (Caspersson, 1950b).

6: "In the production of the not-so-strictly linearly arranged proteins, as in the case of the formation of the cytoplasmic proteins and reproduction of simple viruses ..... the nucleic acids participating are of ribose character." (Caspersson, 1950b).

In addition to the numerous reports from Caspersson and his co-workers, several publications describe qualitative or semi-quantitative observations made with the quartz microscope. Thus Uber (1939) made observations on maize pollen grains, while in 1946 Opie and Lavin, and Davidson and Waymouth, reported on the localisation of nucleoproteins in mammalian liver cells, as shown by ultraviolet microscopy. The latter workers also demonstrated the effect of ribonuclease in reducing the absorption of ultraviolet light by the nucleolus. Pollister and Ris (1947) described the estimation of purine and pyrimidine compounds by ultraviolet absorption measurements in unstained cells: these authors suggested that non-specific light loss could be allowed for, if "difference" measurements were made, before and after removal of U-V absorbing material, e.g., by specific enzymes.

Methods involving the actual calculation of amounts of U-V-absorbing material within particular cell structures have been less widely used than the simpler techniques involving comparisons of the intensity of absorption of U-V light. Caspersson (1939) made calculations of the amount of absorbing material in grasshopper spermatocyte nuclei, based on the dimensions of the specimen, and the average extinction as measured in the quartz microscope. Thorell (1947, 1950) also using photographic methods, calculated the amount of haemoglobin in individual erythrocytes. Walker and Davies (1950) made integrated density measurements on photographs of single cells in living tissue cultures, and were able to compare the total amounts of U-V absorbing material in different nuclei. In 1951, Caspersson, Jacobsson and Lomakka described apparatus for photoelectric estimation of the total absorbing material in cell structures. Walker and Yates (1952a, b) have employed microdensitometer studies on U-V photographs of living tissue cultures, in conjunction with time-lapse cinephotography, to report on the total amount of U-V-absorbing material in the cells in relation to their stage in the mitotic cycle. Leuchtenberger, Leuchtenberger, Vendrely and Vendrely (1952) measured the amounts of DNA in single nuclei of ox liver, thymus and sperm, by ultraviolet absorption measurements on isolated nuclei. These authors,

however, considered it justifiable to calculate the absolute amounts of DNA in single nuclei, basing their calculations on values for the specific extinction coefficient of DNA found for dilute solutions in bulk. They also state that since the absorption due to proteins at 2570 A is only one-fiftieth of that due to nucleic acids at the same concentration and wavelength, and since the concentration of proteins actually present is only about twice to four times that of the nucleic acids, the influence of proteins on U-V absorption at 2570 A can be neglected. Similarly, since the ribonucleic acid (RNA) of the nucleus is present, on average, as about 5% of the total nucleic acids, they consider that its influence on absorption can be neglected. Leuchtenberger, Klein and Klein (1952), however, working with mouse ascites and lymphoma tumour cells, have compared the amounts of nucleic acids as estimated by U-V absorption measurements with the amounts found for the same types of cells in bulk, and find good agreement provided they make allowance for the presence of nuclear RNA, by carrying out U-V absorption measurements after treatment of the specimens with solutions of ribonuclease.

Wyckoff (1952) has described a reflecting U-V microscope used in conjunction with automatic scanning and recording equipment for rapid measurements of integrated U-V light absorption in large numbers of biological specimens,

with a view to adequate statistical analysis of the data obtained. Although from the electronic aspect this instrument is probably the most highly developed pattern yet described, optical difficulties have so far prevented its full exploitation in biological studies.

An extensive discussion of optical methods in the study of cell structure is to be found in the Discussions of the Faraday Society, No.9 (1950).

1.4. Microspectrophotometry in visible light.

1. 4a. Apparatus.

The development of microspectrophotometric techniques in the ultraviolet region by Caspersson acted as a stimulus to the development of similar, but technically less difficult, procedures in the visible region of the spectrum. Probably the first application of quantitative cytochemical methods in the visible region of the spectrum was made by Stowell (1942), who estimated deoxyribonucleic acid in nuclei of tumour cells by photometric measurement of the intensity of Feulgen staining. More general application of microspectrophotometry in visible light, however, began a few years later when Pollister and Ris (1947) described a simplified apparatus for the measurement of colour within single cells. In principle, the apparatus corresponds to that developed by Caspersson (1940, 1950b), except that it is designed for use

in the visible region of the spectrum and therefore uses normal achromatic microscope lenses. A number of instruments have been constructed in other laboratories, differing in detail as regards light sources, arrangement of photoelectric cells and the like, but all corresponding in principle to the Pollister instrument. It is not intended to discuss the various technical modifications in detail.

The original instrument of this type, as used by Pollister and Ris (1947) and described in detail by Pollister and Moses (1949), consists of a standard microscope with mechanical stage, a number of alternative light sources which illuminate the microscope specimen with light of any chosen wavelength, and a focussing screen fitted with a calibrated iris diaphragm just in front of the image plane. There is also a mechanical arrangement for moving aside the focussing screen, and readily replacing it with a sensitive photoelectric cell of the electron multiplier type. The latter is connected to suitable power supplies of high stability, and the output of the photocell is measured by means of a sensitive galvanometer or valve voltmeter.

In practice, a suitably stained tissue section is mounted on the microscope stage, and illuminated with monochromatic light, of a wavelength corresponding as closely as possible to that most strongly absorbed by the coloured substance to be estimated. The structure which one wishes to

measure, e.g., a cell nucleus, is brought into focus in the centre of the focussing screen, and the iris diaphragm is closed down so that it just encloses the nucleus. The photocell is then swung into position, and the intensity of the light passing through the nucleus is recorded in arbitrary units. The specimen is then moved so that the nucleus is replaced by an adjacent clear area on the microscope slide, and a second galvanometer reading is obtained. The ratio of these two readings gives the transmission of the nucleus at that wavelength (provided the output of the photocell, and the galvanometer reading, both increase linearly with increasing light intensity), i.e.,

$$\text{Transmission (T)} = \frac{\text{Intensity of light passing through nucleus}}{\text{Intensity of light originally reaching nucleus}}$$

$$= \frac{\text{Galvo reading with nucleus in position}}{\text{Galvo reading with nucleus removed}}$$

If one assumes that all light lost within a structure such as a stained nucleus is lost through absorption by coloured molecules, then from the transmission value "T", and the dimensions of the nucleus, it is possible to calculate, in arbitrary units, the total amount of light absorbing material which it contains. If one wishes to compare such values for two similarly stained structures, then it is necessary to show that the coloured substance being measured conforms

to the Beer-Lambert laws governing the absorption of light in solutions.

Using an instrument based on these principles, Pollister and Ris (1947) described the measurement of the intensity of Feulgen staining in mammalian cell nuclei, using the measurements as a basis for the calculation of the amounts of DNA within single nuclei. They also estimated amounts of protein in parts of single cells, by photometric estimation of the colour formed within the specimen when treated by a modification of the Millon reaction for tyrosine. Similarly, Pollister and Ris claimed that it was possible to measure the amounts of DNA and RNA within thymus nuclei by quantitative estimation of the amounts of methyl green and pyronin dye taken up during staining with these dyes. In support of this latter technique, they compared the values found for the RNA/DNA ratio for thymus and liver nuclei with the ratios found by chemical estimation on nuclei isolated from these tissues in bulk, and found good agreement. It is a striking warning against the dangers of uncritical acceptance, even of apparently corroborative data, that Pollister (1952) has since admitted that the agreement was entirely fortuitous, and due merely to certain errors in the estimations happening to cancel each other in this particular case. Subsequently, too, Pollister and Leuchtenberger (1949) have admitted that

pyronin staining is by no means selective for RNA, and therefore well-nigh valueless for its photometric estimation.

Despite setbacks due to initial over-enthusiasm, which led to the use of methods whose quantitative validity had not been established, microspectrophotometry in visible light has been extensively used in a number of laboratories, the great majority of the estimations being concerned with the use of the Feulgen reaction for DNA as a means of measuring the amount of DNA in single nuclei, or in chromosomes at different stages of the mitotic cycle. It is impracticable to list here the very large number of reports which have appeared, but contributions of value in the development of the photometric method include those of Ris and Mirsky (1949), who checked the response of their microphotometer by measurements on objects of known optical density; Leuchtenberger (1950), who studied the degenerative process of pyknosis in normal and tumour nuclei in mice, and discussed many of the possible errors in the method; Leuchtenberger, Vendrely and Vendrely (1951), who compared the amounts of DNA found in nuclei from a number of rat and ox tissues with the values obtained by bulk chemical analysis, and found good agreement; Swift (1950), who made extensive studies on the DNA content of mouse nuclei and correlated the results with existing histological knowledge; Moore (1952) who compared the DNA contents of the nuclei of

haploid and diploid frog embryos, finding values in accord with genetical theory; Naora (1951, 1952), who drew attention to the errors possible when minor optical defects are overlooked; and Kurnick (1950a,b) who studied the possibility of applying the methyl green staining technique to the estimation of DNA. Pollister (1952) has reviewed the techniques, validity and applications of visible light microspectrophotometry as applied to cytology, while Swift (1955) has reviewed the field of cytochemical methods in so far as they apply to the nucleic acids.

1.4b. The Feulgen Reaction.

In 1924, Feulgen and Rossenbeck described a staining method which has proved to be of great value in the study of the cell nucleus, both qualitatively and quantitatively, and which is universally known as the Feulgen reaction. Many variations of the test have been described, but in its essentials there are three steps involved,

1) a reagent is prepared, consisting of decolorised basic fuchsin, the decolorising agent being usually one of the salts of sulphurous acid, in an acid medium,

2) the specimen studied, e.g., a tissue section, is submitted to moderate acid hydrolysis,

3) application of the fuchsin reagent to the specimen results in selective staining of cell nuclei, or in some

cases of intranuclear structures. In a satisfactorily stained section, the nuclei are stained deep reddish-purple or magenta, while the cytoplasm remains colourless. The nucleolus is unstained.

The process of Feulgen staining obviously bears a resemblance to the well-known Schiff's reagent test for aldehydes, and the commonly accepted interpretation of the reaction involves the liberation of aldehyde groups during the mild acid hydrolysis of DNA, the aldehyde groupings arising from the deoxypentose residues in the DNA molecule. Any other aldehydes present, but of smaller molecular size than the partially hydrolysed DNA molecule, are presumed to be removed during the hydrolysis and subsequent washing, and the reaction is regarded as specific for the presence of DNA. Support for this interpretation comes from several sources. Thus Brachet (1946) has shown that bisulphite, acting as an aldehyde-blocking agent, inhibits the reaction, while Lessler (1951) has shown a similar effect of hydroxylamine. Similarly, treatment of the specimen with solutions of deoxyribonuclease destroys its ability to stain with the Feulgen reagent. Overend and Stacey (1949) have provided further evidence in support of the aldehyde hypothesis by studies on a range of pentose sugars, which indicate that deoxypentose in the furanose form readily gives rise to aldehyde groupings on acid hydrolysis.

Although the approximate mechanism outlined above has been tacitly accepted by the majority of workers, Stedman and Stedman (1943; 1947; 1950) have strongly advocated an alternative interpretation, not so much of the mechanism of production of the colour, as of the histochemical significance of a positive Feulgen test. They claim that although hydrolysed DNA reacts with the Schiff reagent to give the characteristic colour, the coloured substance is soluble and diffusible, and becomes concentrated on the non-histone protein precipitated in the fixed nucleus. If this interpretation is correct, then a positive reaction given by any cell structure implies the presence in it, not of DNA, but of non-histone protein. In support of this view, the Stedmans have investigated the material liberated by acid hydrolysis of isolated nuclei, and have found that the hydrolysis products in solution do in fact re-colourise Schiff's reagent. It has not, however, been shown that this regenerated dye can specifically stain non-histone protein, nor can it stain structures such as chromosomes, except as part of a generalised faint staining of the whole section (Swift, 1955). Overend and Stacey (1949) have shown that mild acid hydrolysis of DNA, comparable to that involved in the Feulgen process, splits off purine bases from the DNA molecule. The residual product is insoluble in dilute HCl, and therefore would remain in the nuclei in a histological specimen. It does

however, give an immediate and intense reaction with Schiff's reagent, the insoluble product taking on the characteristic colour of Feulgen-positive material. Since these experiments were carried out on purified DNA and not on nucleoprotein, they appear to provide satisfactory evidence that the material which stains in a positive Feulgen reaction in tissues is in fact DNA.

One cannot of course exclude, on this evidence, the possibility that other compounds remaining in the fixed, washed and hydrolysed section might be capable of restoring the colour to the fuchsin reagent, and it is therefore desirable that in every case a control specimen should be stained without prior hydrolysis, in order to reveal any non-specific staining. It is also preferable to submit a control specimen to the action of deoxyribonuclease prior to hydrolysis and staining, when positive reactions due to compounds other than DNA should become obvious. If these precautions are taken, then the specificity of the reaction for DNA can be regarded as satisfactorily established.

Although a properly carried out Feulgen test provides evidence of the presence of DNA within a cell structure, a negative test is less satisfactory as evidence of its absence. In certain oocyte nuclei, for example, a negative reaction is found, although the presence of deoxypentose compounds can be detected chemically. In these cases, the

nucleus is invariably very large, and one may reasonably regard the absence of detectable Feulgen-positive material as indicating that the DNA of such nuclei is dispersed through the nucleus at low concentration.

Where it is intended to employ the Feulgen reaction not only to show the presence of DNA, but also as the basis of quantitative photometric work for the estimation of amounts of DNA within single nuclei it is obviously essential to know what relationship, if any, exists between the amount of colour produced in the nucleus and the amount of DNA originally present. Lessler (1951) constructed model "nuclei" consisting of drops of gelatin containing DNA in various concentrations, and found a linear relationship between dye intensity and DNA concentration up to about 1 mg. DNA per ml. of gelatin. Since this concentration is considerably below that occurring in animal nuclei, further evidence is necessary before accepting quantitative Feulgen dye measurements as valid for the estimation of DNA. On the other hand, Ris and Mirsky (1949), Swift (1950) and a number of other workers, when measuring the Feulgen dye content of nuclei from organs such as liver, which are known to contain a proportion of diploid, tetraploid, and octaploid nuclei, found the dye content of the nuclei to fall into three distinct classes, corresponding almost exactly to the values one would expect on the assumption

that a single chromosome set contains a definite amount of DNA. Ris and Mirsky have also shown a good correlation between the DNA content of nuclei as measured in bulk by chemical methods and on single nuclei by Feulgen staining and photometry. Swift (1950a,b) has also shown that the Feulgen dye retained by nuclei in several mouse tissues is the same, within the errors of measurement, but is totally different from that in tissues of other species, the differences being closely related to the relative amounts of DNA in such nuclei as measured by chemical methods.

Valuable indirect evidence concerning the relationship between chromosome sets and Feulgen dye intensity comes from the observations of Moore (1952) on haploid and diploid frog embryo nuclei, in which the amounts of Feulgen dye again corresponded to the presumed relative DNA content based on the assumption of a constant amount of DNA per chromosome set within a single species. Nevertheless, it cannot be regarded as finally proved that the Beer-Lambert laws are fully valid within the concentration range for DNA found in animal nuclei. The most that can be said is that discordant evidence has been found in respect of gelatin-DNA mixtures, whereas in almost all cases where a range of nuclei, of known mean DNA contents, has been compared in side-by-side estimations of Feulgen dye content, the results agree very well indeed with what would be expected if the

Beer-Lambert laws apply to this particular system. (The influence of variations in Feulgen technique, fixation, etc., will be discussed later.) It is hoped in the present series of experiments to provide further evidence on the validity of the Feulgen-photometric technique.

1.4c. Methyl green staining.

It has been known to histologists for many years that basic dyes show a high affinity for nuclear chromatin, and that when a tissue section is first generally stained by such dyes, and then "differentiated", that is, treated with a rinsing solution intended to remove unbound dye, the nuclei retain the dye to a much greater degree than does the cytoplasm, although prolonged differentiation may ultimately remove all of the dye from a section. It is not intended to discuss in detail the many factors affecting basic dye binding, but it should be noted that the major factor affecting the affinity of a nuclear structure for basic dyes is the electrostatic attraction between the negatively charged phosphate groups on the DNA molecule and the positive charge carried on the dye molecule. The amount of staining produced and retained will therefore be critically dependent on the degree of dissociation of both dye and nucleic acid, and therefore will be greatly influenced by the pH of the staining and rinsing solutions. If,

however, suitable conditions could be found whereby a reproducible relationship exists between the amount of DNA present, and the amount of basic dye with which it is combined, then such a system could be used for the estimation of DNA within a nucleus.

Of the range of basic dyes available for nuclear staining, only one has found application in the quantitative study of DNA in the single nucleus, namely methyl green. Kurnick (1950a,b; 1952), Kurnick and Mirsky (1950), Kurnick and Foster (1950), Kurnick and Herskowitz (1952) have extensively studied the interaction of methyl green with DNA, both in the tissue section and <u>in vitro</u>, and have concluded that under suitable conditions methyl green staining is a valid basis for the photometric estimation of DNA. Kurnick considers, however, that the selective staining of DNA with methyl green, while RNA remains colourless, is related to its greater molecular size, and has shown that treatment which depolymerises the DNA results in loss of stainability with methyl green. (Somewhat similar findings in regard to acridine binding by DNA and RNA have been reported by Irwin and Irwin (1949, 1952). Taft (1951), however, has put forward an opposing view, having failed to influence methyl green staining by prior exposure of DNA to conditions of temperature and pH which are known to cause depolymerisation. Kurnick (1952) has disputed Taft's views, suggesting

that the conditions which Taft employed do not cause irreversible depolymerisation, and also that some of the discordant results are explicable on the basis of the use of partially degraded DNA at the start of the experiments.

Unlike the majority of workers using Feulgen staining for photometry, Kurnick has claimed to be able to use extinction coefficients found for the DNA-methyl green complex *in vitro* as a basis for the calculation of absolute values of DNA for single nuclei.

1.5. The Nucleoproteins - Identification and Structure.

Early knowledge of the composition of the cell nucleus dates from the latter half of the nineteenth century, when Friedrich Miescher investigated the components of animal nuclei from a variety of sources. In 1871, he reported on experiments performed in 1868, in which nuclei were obtained from pus, following digestion of the cytoplasmic material with a synthetic gastric juice. Extraction of the nuclei with dilute solutions of sodium carbonate, followed by acidification of the extract, yielded a somewhat sticky precipitate, which could be redissolved in alkali and reprecipitated by acid. The material was called "nuclein", and was shown by Miescher to contain protein and, somewhat surprisingly, phosphorus. Similar compounds were isolated within a few years, by Hoppe-Seyler (1871) from yeast, and

by Kossel (1882) from nucleated avian erythrocytes. At the present time these "nucleins" would be classed as impure nucleoproteins.

Miescher continued his nuclear researches by investigating the composition of salmon sperm, which by treatment with acetic acid can be made to yield sperm heads, having little, if any, cytoplasmic contamination. Miescher (1874a,b) found that the principal constituent of salmon sperm heads is a salt-like compound of a nitrogenous base, protamine, with a phosphorus-containing acidic compound, nuclein (or nucleic acid, as it was subsequently named by Kossel).

The structure of nucleic acids and their complexes with proteins was further investigated by Piccard (1874), who showed that the nucleins of Miescher contained nitrogenous bases of the purine type; they were therefore quite distinct from the proteins. Kossel extended this work greatly between 1880 and 1903, identifying the purines adenine and xanthine, and also the pyrimidine bases cytosine and thymine (Kossel, 1881, 1896; Kossel and Steudel, 1902, 1903). Other workers who made outstanding contributions to the identification of breakdown products of the nucleic acid molecule include Hammarsten, Jones, Levene and Mandel. Their contributions have been brought together and summarised in monographs by Jones (1914), by Feulgen (1923), and by

Levene and Bass (1931).

It should be remembered that with the exception of Miescher, the early nucleic acid investigators used somewhat drastic methods for the isolation of their compounds from the cell, e.g., Neumann (1898) used hot alkali as the extracting agent. It is now well known that such treatment can only yield a highly degraded product, and while that may even be of advantage in qualitative studies on molecular components, it must inevitably give a false idea of the complexity of the nucleic acid molecule. One result of these drastic treatments was to encourage the erroneous idea that "nucleic acid" represented a single substance, since the same degradation products were obtained from nucleins from a variety of sources. In 1914 Jones declared that there are only two nucleic acids, one derived from animal sources and the other from plants, including yeast. Degradation products of plant nucleic acid, or yeast nucleic acid as it has commonly been called, were given by Jones as phosphoric acid, guanine, adenine, cytosine, uracil, and pentose. Animal nucleic acid, or thymus nucleic acid, on the other hand, was shown on hydrolysis to give rise to phosphoric acid, guanine, adenine, cytosine, thymine, and laevulinic acid, the latter being said to be derived from a hexose in the thymus nucleic acid molecule. So little was the possibility of species differences considered,

that even the species of origin of the nucleic acid was omitted when presenting analytical data (Levene and Jacobs, 1912). To quote from Jones (1914) "..... all nucleic acids are identical with one or the other of these two compounds. This opinion is based upon the most firmly established facts in our possession .....". Another well known worker, Steudel (1908), suggested that the designation of animal nucleic acids by the names of the glands from which they are obtained is as superfluous as would be the application of similar nomenclature to a substance such as lecithin.

Apart from the difference in the pyrimidine components of plant and thymus nucleic acids (to use the old nomenclature), the most obvious difference lay in the carbohydrate portion of the molecule. In 1909, Levene and Jacobs identified that derived from hydrolysis of yeast nucleic acid as D(-) ribose, and thymus nucleic acid (from the thymus gland) was also shown to contain a pentose sugar which was identified by Levene and London (1929) as D-2-deoxyribose. No other deoxypentose has so far been identified in any nucleic acid, despite examination of numerous samples by chromatographic methods (Chargaff and Lipshitz, 1953).

By 1931, it had become clear that portions of the nucleic acid molecule could be isolated by partial hydrolysis in the form of larger units known as nucleosides and

nucleotides, the former containing one molecule of either a purine or a pyrimidine base and one pentose or deoxypentose sugar residue, while the nucleotides were shown to be phosphoric esters of the nucleosides. The nucleic acid molecule was regarded as a condensation product of nucleotides, the exact mode of linkage and the overall molecular size being as yet uncertain.

The early dogma of the sharp distinction between pentose nucleic acids from plants, and deoxypentose nucleic acids from animal sources, was by no means universally accepted. Jorpes (1924, 1928), for example, succeeded in preparing a pentose nucleic acid from pancreas, while several other workers obtained pentose compounds from animal tissues. Also, Feulgen and Rossenbeck (1924) obtained histochemical evidence for the occurrence of deoxypentose nucleic acids in plant nuclei, the final proof being given in 1948 by Chargaff and Zamenhof, who isolated a nucleic acid from wheat germ which they proved to contain deoxypentose and thymine, i.e., to be a typical deoxypentose nucleic acid. It is therefore clear that neither plants nor animals have a monopoly of either type of nucleic acid, and the terms "plant nucleic acid" and "animal nucleic acid" no longer have any meaning.

Early analytical studies suggested that the two purine and two pyrimidine bases found in nucleic acids might be

present in equimolecular proportions, suggesting the possibility that nucleic acids might have a tetranucleotide structure (Feulgen, 1918). Jones (1920) also proposed a tetranucleotide, suggesting that the linkage between adjacent nucleotides might be between the sugar portions of the nucleotides. More recently, Greenstein (1944) suggested that in fact the nucleic acids, both DNA and RNA, consist of polytetranucleotides, each tetranucleotide containing one of each of the four constituent nucleotides.

The idea of the tetranucleotide received some support from the early attempts to measure the molecular weight of nucleic acid. Thus Myrback and Jorpes (1935) found values of around 1500, which, allowing for the crudities of the techniques then available, was in reasonable agreement with the theoretical value for a tetranucleotide, approximately 1550. More recently, however, it has become clear that Myrback and Jorpes were working with partially degraded material, and that their results are therefore invalid. Further, Fletcher (1948) recalculated their data, and claims that their material had, in fact, a molecular weight of about 6000. Had this value been published in 1935, the simple tetranucleotide theory might have been discredited at a much earlier date. Many studies within the past few years, however, have indicated that the values found by early workers are much too low. No agreement appears to

have been reached as to which of the many published values is correct, but for deoxypentose nucleic acids most workers agree that the true value is measured in millions rather than in thousands. For calf thymus DNA, for example, measurements of the sedimentation and diffusion constants give values of the order of 1.3-1.5 x 10^6 for the molecular weight. (Cecil and Ogston, 1948; Conway, Gilbert and Butler, 1950).

Light-scattering measurements, however, even on the same samples as have been used for sedimentation constant measurements, indicate much higher values, of the order of 4-8 x 10^6 (Smith and Sheffer, 1950; Katz, 1952). By contrast, values found by dielectric constant methods are much lower, e.g., Jungner (1950) has found values of 1.2-8.4 x 10^5. The reasons for the discrepancies are not clear, but it seems likely that the different methods are measuring the size of particles differing in their physical behaviour, e.g., the unit which orientates in an electrostatic field may readily differ from the unit which sediments under gravity. In macromolecules of this character, the concept of a pure substance consisting of very large numbers of identical molecules ceases to have the clear-cut significance which it has in the case of small molecules. Whether these huge macromolecules of purified DNA do in fact correspond to the molecular size of DNA within the living

cell is impossible to determine - they might, for example, be artefacts, arising by aggregation during the process of preparation. Doty and Bunce (1952), however, conclude from the shapes of their light scattering curves that no aggregation is present. Their assessment, however, is apparently based on the assumption that aggregation would be reversible on dilution. This need not necessarily be true, particularly if a large number of similar but not identical molecules can aggregate, and disunite at different linkages from those which formed during the aggregation.

In the case of the molecular weights of the pentose nucleic acids, technical difficulties in the isolation processes make the preparation of satisfactory samples difficult, especially in view of the probability of heterogeneity in the preparations. Probably the most reliable values are those of Cohen and Stanley (1942) for tobacco mosaic virus which suggest about 6×10^4 as the probable value. Volkin and Carter (1951) and Kay and Dounce (1953) have found values of the order of 10^5 for animal RNA.

The manner in which the constituent nucleotides are built up to form the macromolecules of nucleic acid remains somewhat speculative. The tetranucleotide hypothesis already mentioned could not be maintained in the face of molecular weight determinations, while the idea of a polymerised tetranucleotide rested on the assumption that all four

constituent bases are present in exactly equimolecular proportions, and also that the bases occur in the same order from end to end of the long fibrous molecules of DNA. If this were so, the only differences between nucleic acids from different species or tissues would lie in the molecular sizes, and possibly in the manner of union of adjacent nucleotides. Gulland and his co-workers, however, pointed out that equimolecular proportions of bases might still be consistent with innumerable possible variations in the sequence of the nucleotides within a molecular chain. Thus arose the concept of the statistical polynucleotide (Gulland, Barker and Jordan, 1945; Creeth, Gulland and Jordan, 1947).

Improved analytical methods, and especially the quantitative application of paper and column chromatography, have clearly shown that the idea of four bases occurring in exactly equimolecular proportions in the nucleic acid molecule is no longer acceptable. Gulland, Jordan and Threlfall (1947) showed that the $\frac{\text{purine N}}{\text{pyrimidine N}}$ ratio in both calf thymus DNA and yeast RNA is too low to fit the tetranucleotide hypothesis, while Hotchkiss (1948), Vischer and Chargaff (1948) and Markham and Smith (1949) confirmed that the purine and pyrimidine bases are not present in exact equimolecular proportions. Chromatographic methods, also, have shown that in herring sperm DNA and in bacteriophage DNA additional pyrimidine bases occur which have been

identified as 5-methylcytosine and hydroxymethylcytosine respectively (Wyatt, 1951; Wyatt and Cohen, 1952). 5-Methylcytosine occurs most richly in wheat germ DNA, but small amounts have been found in animal DNA samples also. It is not improbable that other bases, or possibly unexpected nucleotides or nucleosides, may yet be found. Enough has been said to indicate that the tetranucleotide hypothesis is not now tenable.

Many workers, using chromatographic techniques, have studied the proportions of the various bases or nucleotides found in hydrolysates of DNA and of RNA from a variety of sources, both plants and animals, as well as microorganisms and viruses. The results are not entirely consistent between different workers, and it appears that the technique of isolation of the nucleic acid, the manner of hydrolysis, and the means used to separate the nucleotides or bases, may all affect the final yield of each, so that published values must still be regarded as approximate. Collected lists of analytical findings have been made by Chargaff (1955) and by Magasanik (1955). While it is difficult and perhaps unwise to generalise from the data at present available, it appears true to say that within a particular animal species, the DNAs from different tissues do not differ greatly in the proportions of the four bases, whereas there are considerable differences in the proportions of bases in

RNA samples obtained from various organs, and indeed in the RNA associated with the nucleus as compared with that from the cytoplasmic particles within one tissue. Slightly greater differences are found between samples of DNA obtained from the same organ in different animal species, while extension of studies to microorganisms shows considerably greater differences. Chargaff (1951) and Chargaff, Zamenhoff, Brauerman and Kerin (1950) have calculated the ratios of bases found in DNA samples from different sources, and show that the ratios adenine/thymine and guanine/cytosine are fairly close to unity. They also point out that in all animal and some microbial DNAs, adenine and thymine are predominant (the "AT" type) while in some microbial DNAs, guanine and cytosine predominate (the "GC" type). Whether these findings have any bearing either on the functions of DNA or on the problem of molecular architecture is uncertain, but it is interesting to note that Chargaff, Crampton and Lipshitz (1953) have found that when DNA is dissociated from protein by increasing concentrations of sodium chloride, there is a progressive increase in the adenine/guanine and thymine/cytosine ratios in the DNA liberated, while preserving the normal adenine/thymine and guanine/cytosine ratios. Although such physico-chemical effects of the differences in base ratios may be widespread, there is as yet no information as to the biological

importance, if any, of such variations.

The ribonucleic acids vary much more widely in composition than do the deoxyribonucleic acids, even within the tissues of a single animal (Chargaff, 1950), and indeed there is considerable evidence that nuclear RNA may differ considerably from the cytoplasmic RNA derived from the same cells. (McIndoe and Davidson, 1952; Elson and Chargaff, 1952; Crosbie, Smellie and Davidson, 1953.)

Even where the ratios of the bases are reasonably constant, it must be remembered that what has been estimated is the mean composition of all the molecules in the nucleic acid sample from which the hydrolysate was prepared. Even a so-called highly purified nucleic acid sample might very well consist of a large number of chemically related but slightly different molecules, in which case the constancy of ratios of bases in hydrolysates would merely reflect some degree of stability in the population of molecules from which the sample was obtained. In respect of DNA, evidence has been accumulating in recent years which strongly suggests that purified DNA represents a mixture of several, if not many, distinct substances, differing in physico-chemical properties, and possibly also in biological activity (Stern, 1952; Bendich, Russel and Brown, 1953; Barton, 1952; Chargaff, Crampton and Lipshitz, 1953; Brown and Watson, 1953). These distinct DNAs may each represent

many chemically similar molecular species, of similar metabolic origin, yet differing in their biological roles. As yet, chemical methods are quite unable to resolve such mixtures, except by the insensitive criterion of base ratios, while biological methods for their recognition hardly exist.

The manner of construction of the nucleic acid molecule from its constituent nucleotides has engaged the attention of many workers. The fibrous nature of purified DNA, together with its very high viscosity in aqueous solutions and such optical properties as streaming birefringence, all suggest greatly elongated molecules. Since pure preparations of RNA of high molecular weight are not readily obtainable, the great majority of structural studies have been made on the sodium salt of deoxyribonucleic acid, particularly that from calf thymus. Astbury and Bell (1938), on the basis of X-ray diffraction diagrams obtained from solid samples of DNA, and of density measurements on dried DNA, concluded that the DNA molecule consisted of a stack of planar nucleotides arranged perpendicular to the fibre axis. They also indicated that the structure contained a pattern which repeated at intervals of 27A along the fibre. Studies by Furberg (1952), however, showed that some at least of the nucleotides do not form planar units. An alternative structure was proposed by Pauling and Corey (1953) involving

three intertwined helical chains of nucleotides, with the phosphate groups directed towards the centre of the spiral, and the purine and pyrimidine rings on the outside. This structure, however, is not consistent with the titrimetric behaviour of the DNA ion (Gulland, Jordan and Taylor, 1947), nor with its well-known affinity for basic dyes. In 1953, Watson and Crick proposed a different type of spiral structure, consisting of two parallel helical chains of nucleotides, arranged in opposite directions, and united by hydrogen bonds between purine and pyrimidine bases. Stereochemical considerations suggest that only adenine could form suitable bonds with thymine, while guanine could form the necessary linkages with cytosine, thus providing a possible basis for the observations of Chargaff (1950) and others on the molar ratios of adenine/thymine and guanine/cytosine, mentioned earlier in this section. At the present time, this double helix structure appears to be in good accord with the available information on DNA molecular structure.

In regard to RNA, no equivalent information has been obtained. There are, however, indications, based on enzymic degradation studies, that the RNA molecule may have a multiple branched chain structure totally unlike that of DNA (Volkin and Cohn, 1953).

The final step in the determination of the structure

of the long-chain nucleic acid molecule must be the determination of the sequence of the nucleotides in the chain (and in addition, the determination of the presence or absence of breaks or branching in either of the nucleotide helices). Mild acid hydrolysis of DNA yields a material of relatively high molecular weight, but containing no purines, which has accordingly been named "apurinic acid" (Tamm, Hodes and Chargaff, 1952). Studies of the products of further degradation of this material may give some indications of pyrimidine nucleotide sequences in relation to the deoxypentose phosphates which presumably take the place of the original purine nucleotides. The prospects of obtaining a complete map of nucleotide sequences in any single DNA do not at the present time appear hopeful. In any case, the study of degradation polynucleotides is likely to lead to confusion unless the original preparation of DNA is homogeneous as regards the molecules which it contains, a situation which cannot be guaranteed for any preparation at the present time.

1.6. Nucleic acids - localisation within the cell.

Nucleic acids form a characteristic and essential constituent of living cells, and of the class of infective particles commonly called viruses. They were, of course, given the name "nucleic acids" at a time when they were

considered to be confined to the cell nucleus, and some years elapsed before it was suspected that substances of this class occurred also in the cell cytoplasm in significant amounts (Beebe and Shaffer, 1905). The actual sites within the cell of DNA and RNA, however, were recognised by means of specific histochemical tests. The Feulgen reaction (Feulgen and Rossenbeck, 1924) indicated quite clearly that DNA is confined to the cell nucleus, both in plants and in animals. It was not therefore surprising that chemical estimations of DNA showed it to be present in high concentration in tissues whose nuclear content is high, e.g., organs such as spleen, thymus, lymph glands, but in relatively low concentration in tissues having a high proportion of cytoplasm, such as brain. (In discussing the nucleic acid content of tissues, one must distinguish between concentration, which implies the amount of nucleic acid per unit wet or dry weight of tissue, and nucleic acid content of a tissue, which is usually taken to mean the total amount of the nucleic acid contained in a particular organ of a particular animal. It is obvious that, for example, changes in the fluid content of tissues may greatly alter the concentration of a nucleic acid within a particular organ, without signifying any metabolic change in the cells with respect to nucleic acids. Similarly, the nucleic acid content of a particular organ may vary considerably from one animal to another, without implying

that the animals necessarily differ except in the size of the organ. It is therefore highly desirable to have some standard in terms of which the amount of nucleic acid may be expressed, as, for example, per unit of protein nitrogen or per unit of total nitrogen. Even these may be misleading if there is a simultaneous variation in the cell's content of protein and of nucleic acid. The ideal method of expressing the amount of a cell constituent would be in terms of the absolute amount of the dry constituent present in a single cell, if that were possible. It is, however, rarely possible to do so by present methods, except where the cell population is almost homogeneous and is easily counted, as for example in the case of erythrocytes. In solid tissues, difficulty arises due to variation from cell to cell, as well as to difficulty in counting the number of cells with sufficient accuracy.)

Evidence that DNA is not found outside the cell nucleus, based on the Feulgen reaction, is not in itself conclusive, since DNA might well be present in amounts too small to detect by this means, or present in some "combined" form, non-reactive as regards the Schiff reagent. The general hypothesis, that DNA is a nuclear constituent, has been confirmed by many workers, using techniques of cell fractionation which effectively separate nuclei from cytoplasmic material (Schneider, 1946, 1948; Hogeboom,

Schneider and Palladе, 1948; Schneider and Potter, 1949; Price, Miller and Miller, 1948; Petermann, Slater and Larack, 1949). However, important exceptions have been recorded, though they do not appear to invalidate the generalisation. Thus Sparrow and Hammond (1947) have shown that a few types of plant cells contain cytoplasmic bodies giving positive reactions for DNA. Hoff-Jorgensen and Zeuthen (1952) have detected deoxypentose compounds in the cytoplasm of frog eggs, while Fraenkel-Conrat, Snell and Ducay (1952) have also found similar compounds in the avidin fraction of egg white. Whether these substances represent material of high molecular weight, similar to the DNA found in nuclei, or whether they represent deoxypentose nucleosides or possibly oligonucleotides, has not been fully established. This subject has been reviewed by Hoff-Jorgensen (1954). In the great majority of cases, however, cytoplasmic material, when carefully freed from nuclei or nuclear fragments, contains no significant amount of highly polymerised DNA.

The localisation of ribonucleic acid within the cell has been studied by three main methods, the first being that of ultraviolet light absorption studies, already referred to in Section 1.3. This technique does not of course prove that the component which absorbs U-V light is always RNA, but the second method, that of Brachet, yields results which

amply confirm those of Caspersson and his school. Brachet (1940, 1941) compared the basophilic staining properties of cells and tissue sections, before and after treatment with a specific depolymerising enzyme, ribonuclease, and showed that cytoplasmic basophilia can be virtually abolished by the ribonuclease digestion. Provided the enzyme removes no cell component other than RNA, this test can readily reveal the localisation, and within limits the amount of RNA contained in the cell. Early preparations of crystalline ribonuclease, prepared according to Kunitz (1940) showed appreciable proteolytic activity, but McDonald's (1948) modified procedure yields a product of high specificity. A number of alternative staining procedures may be used in conjunction with the ribonuclease test, perhaps the most widely used being the combined pyronin-methyl green stain as used by Brachet. The application of this test to many tissues has shown that basophilic cytoplasmic granules are the site of RNA in high concentration, and it has become clear that the nucleolus also contains RNA in appreciable amount. These findings are in agreement with those obtained by ultraviolet microscopy.

The third method for the localisation of RNA within the cell consists in the mechanical fractionation of cells in bulk, firstly by mechanical disintegration of the tissue, followed usually by differential centrifugation of cell

components in suitable media (Claude, 1943, 1946; Crossman, 1937; Mirsky and Polister, 1946) or by differential sedimentation in non-aqueous media (Behrens, 1938; Dounce, Tishkoff, Barnet and Freer, 1950; Allfrey, Stern, Mirsky and Saetren, 1952). The various fractions obtained, frequently designated as nuclei, large granules or mitochondria, small granules or microsomes, and cell sap or non-granular fraction, are then subjected to chemical analysis for nucleic acids by methods such as those of Schmidt and Thannhauser (1945), Ogur and Rosen (1950), Schneider (1945). This procedure has the merit of providing information on the actual concentration of RNA within particular cell components, and of allowing the detailed study of its composition and metabolic behaviour in the various fractions. It is, of course, open to two objections, firstly that each fraction may be contaminated to a serious extent with material derived from the others, the extent of the contamination being difficult to assess, and secondly that the measured concentrations of components in each fraction represent only the mean values for material derived from a very large number of cells, which even in a homogeneous tissue may differ greatly from one to another.

The composition of the various cytoplasmic fractions varies somewhat, according to the procedure for their isolation (cf. Schneider, 1946, 1948), but in rat liver, for

example, the percentages of the total cell RNA (measured as RNA phosphorus) occurring in the various fractions are given by Schneider (1948) as follows:- nuclei, 14%; mitochondria, 7%; microsomes, 52%; and cell sap, 22%. The difficulty of defining the limits of the particle classes makes precision in this field impracticable, but the distinction between the fractions is sufficiently definite to permit of comparison between the RNAs from the four main fractions as regards their composition, as well as their metabolic behaviour. It has been shown that the three cytoplasmic fractions of rat liver yield ribonucleic acids which differ little in the molar proportions of the bases which they contain, though they can be readily differentiated from the RNA obtained from nuclei from the same tissue (Crosbie, Smellie and Davidson, 1953). That nuclear RNA is also metabolically distinct from the cytoplasmic RNA has been shown by its more rapid uptake of a radioactive tracer such as ^{32}P (Smellie, McIndoe, Logan, Davidson and Dawson, 1953); such a finding effectively disposes of the argument that nuclear RNA is merely contaminating cytoplasmic material.

1.7. The composition of the animal cell nucleus.

It has been known to cytologists for many years that the nucleus of a typical non-dividing animal cell is a discrete body, differing in refractive index from the

cytoplasm which surrounds it, and contained within a definite nuclear membrane. Distinct structures are not usually visible within the living nucleus, with the exception of the round nucleolus, but fixation and staining reveal the presence of basophilic bodies, irregular in shape and size, which collectively are termed the chromatin. From its staining reactions, and the fact that it becomes visible after treatment with fixatives, it is generally regarded as consisting mainly of precipitated nucleoprotein. The question of whether chromatin merits the status of being recognised as intranuclear structure, or whether its appearance is to be regarded as a mere fixation artefact, has been a controversial topic between histologists and cytochemists for some time. That there is apparently some relationship between the structure of the chromatin and the genetic constitution of the cell has been demonstrated by the successful determination of chromosomal sex in epithelial cells and in leucocytes, based on the presence or absence of recognisable particle shapes in the chromatin (Moore, Graham and Barr, 1953; Davidson and Smith, 1954).

In addition to the nucleic acids, DNA and RNA, the nucleus invariably contains protein in considerable amount. Miescher in 1868 reported that salmon spermatozoa contained a basic nitrogen-containing compound which he termed "protamine". Kossel later (1884) recorded a somewhat

similar compound in the nuclei of bird erythrocytes and named it "histone". It was shown to be a protein. Some ten years later, Lillienfeld showed that an essentially similar protein was obtainable from thymus gland. The foundations of our knowledge of these compounds were laid by Kossel, who studied the basic proteins of many varieties of cell, and established, firstly, that all nuclei which he studied contained a basic protein, apparently united to nucleic acid by a salt linkage, and yielding, on hydrolysis, comparatively few amino acids. The simpler basic proteins, the protamines, are confined to fish sperm, while histones occur in some fish sperm, e.g., the cod, and in the nuclei of the somatic tissues generally. The question of what constitutes a protamine and what a histone is determined somewhat arbitrarily by reference to the size and complexity of the molecules, the smaller and simpler basic proteins being regarded as protamines while the others form the histones. As Kossel himself pointed out (Kossel, 1928), there may well be several proteins intermediate in character, which could be assigned to either class. The protamines are the simplest proteins so far recorded, with molecular weights of the order of 2,000-10,000. In general, they contain a high proportion of arginine, which accounts for their predominantly basic character. Their relative simplicity, both in molecular size and in the amino acids which

they contain, makes them most unlikely to be the vehicle for transmission of numerous hereditary characteristics by the spermatozoon.

Although Kossel had demonstrated histones in a number of tissues, our knowledge in this field has been considerably extended during the past decade by Mirsky and his coworkers (Mirsky, 1943; Mirsky and Pollister, 1946), and by Stedman and Stedman (1944, 1951). The former group of workers have extracted histones from many animal cells, including mammalian tissues, in the form of their complexes with nucleic acids, the nucleo-histones. The Stedmans, on the other hand, have extracted all the basic proteins from a number of animal and fish tissues, by the simple but relatively drastic procedure of mincing the tissues and extracting with dilute mineral acids, and have shown that there are definite differences in composition between the histones extracted from different tissues in the same animal (Stedman and Stedman, 1947). They have also shown that a single tissue (cod sperm) can provide at least two histones, differing in amino acid composition, which they have designated "main histone" and "subsidiary histone" (Stedman and Stedman, 1951). Further fractionation of histone by electrophoretic means has confirmed the heterogeneity of the basic protein within a single tissue, and has shown differences in the histone composition of the nuclei from

normal and neoplastic tissues.

The histones or protamines, together with the nucleic acids, are not sufficient to account quantitatively for the known mass of the nucleus, but it was not till 1942 that other nuclear proteins were recognised chemically (Mayer and Gulick). These contained appreciable amounts of sulphur, and in general resembled globulins. Caspersson (1941) had found from his ultraviolet absorption studies that proteins, having a higher content of cyclic amino acids than the histones, could be detected in the chromosomes, and appeared to be related to their chemical functions. In 1943, Stedman and Stedman reported that by first removing the basic proteins with dilute acid, then separating the nucleic acids from the residue by means of alkali, they obtained an acidic protein having a high content of tryptophan, as well as tyrosine, glutamic acid, aspartic acid, and the basic amino acids arginine, histidine and lysine. From its staining reactions, the Stedmans concluded that this protein is the nuclear constituent which takes up the dye in haematoxylin staining, and they have regarded it as the principal constituent of the chromosomes. They accordingly named it "chromosomin". Since it has proved difficult to extract this protein quantitatively, the Stedmans have estimated its amount in various types of nuclei by assuming that apart from lipids, nucleic acids, and histones or protamines, all

the dry weight of nuclei is made up of chromosomin. On this assumption, they find that from 33-72% of the dry weight of nuclei is accounted for by chromosomin (Stedman and Stedman, 1943, 1947, 1951).

Some confusion of terminology arose in 1946, when Mirsky and Pollister used the term "chromosin" to describe a nucleoprotein complex which they obtained by extracting nuclei with M NaCl solutions. The name "chromosin" has since been abandoned, but it is possible to obtain from this nucleoprotein complex a mixture of proteins, by dissociating them from nucleic acid using a mixture of chloroform and octanol. The histone can then be extracted by dilute acid, leaving a denatured protein residue similar in its amino acid composition to the "chromosomin" of the Stedmans. Jeener (1946, 1947) has also described a nuclear protein resembling these non-histone proteins, while more recently acidic proteins have been described as constituents of the nuclei of rat liver, calf thymus, calf liver, ox spleen and chicken erythrocytes (Wang, Kirkham, Dallam, Mayer and Thomas, 1949; Wang, Mayer and Thomas, 1953). These workers, although regarding their protein as analogous to "chromosomin", have shown that it contains cholesterol and phospholipids, and they consider that it probably arises from the nuclear membrane rather than from the chromosomes. Blumel and Kirby (1948), however, have

investigated the composition of isolated giant salivary chromosomes from Drosophila, and conclude that the chromosomes themselves contain higher proteins.

The nuclear enzymes, presumably contained in the protein mixture usually designated as non-histone nuclear protein, have attracted considerable interest, especially since the enzymatic functions of the nucleus are highly relevant to its role in the overall metabolism and functions of the living cell. In this field, however, the greatest difficulties are, firstly, to obtain nuclei free from contamination by cytoplasmic material, remembering that the microscopic appearance of the preparation is quite inadequate as a criterion of freedom from cytoplasmic proteins; and secondly, to ensure that nuclear enzymes are not significantly inactivated during the processes of cytoplasm removal. The usual "nuclear fraction" obtained by disruption of a tissue followed by differential centrifugation is so grossly contaminated by cytoplasmic material that studies revealing the presence of enzymes cannot be regarded as establishing that such enzymes are nuclear in origin, unless a very high proportion of the enzyme activity of the whole tissue can be accounted for in the nuclear fraction. Schneider, Hogeboom and Ross (1950), for example, consider on this basis that the adenosine phosphatases of mouse liver are very largely nuclear enzymes, while Novikoff and his

co-workers have reached similar conclusions regarding rat liver (Novikoff, Podber and Ryan, 1950; Novikoff, Hecht, Podber and Ryan, 1952). Dounce (1950) has studied the enzymic abilities of isolated rat liver nuclei, and has shown that a number of enzymes, but particularly alkaline phosphatase, are present in the isolated nuclei in amounts greater, per unit of dry weight, than in the whole tissue. A number of enzymes concerned in glycolysis were found, but no evidence for the presence of the component enzymes for the tricarboxylic acid cycle. On this basis, however, the concept of the nucleus as being purely an inert storehouse for genetic material is hardly justified.

Further support for the idea of the nucleus as an enzymically active structure has come from studies by Stern, Mirsky, Allfrey and Saetren (1952) who have found, in nuclei isolated in non-aqueous media, considerable differences in enzyme activity from tissue to tissue, e.g., in calf tissues, liver nuclei contain catalase while kidney nuclei apparently do not. They also found that fasting alters the distribution of enzymes as between nucleus and cytoplasm, a finding difficult to explain if "nuclear enzymes" were only an expression of some degree of cytoplasmic contamination.

Although it may be safely assumed that the greater part of the dry weight of the cell nucleus is accounted for

by nucleic acids, histones (or protamines), non-histone proteins, including "chromosomin", and nuclear lipids, comparatively little is known regarding other constituents present in lesser amounts. Molecules smaller than those of the nucleic acids and the proteins are unlikely to remain within the nucleus during the processes of isolation in aqueous media, while direct histochemical tests applied to microscope specimens may well be invalid, even on freeze-dried preparations, owing to diffusion resulting from the concentration gradients set up during dehydration, however rapidly the freezing and drying may be performed. The lack of reliable methods for study is mainly responsible for the apparent lack of interest in other than macromolecular nuclear constituents. Some information, however, is available on the mineral content of nuclei isolated in non-aqueous media. Gulick, for example, has found that in lymphocyte nuclei over 1% of the dry weight is accounted for by calcium, while much smaller, but readily detectable amounts of magnesium are present (Williamson and Gulick, 1942; Gulick, 1946). Dounce and Bayer (1948), on the other hand, have reported much lower concentrations of calcium in rat liver nuclei. Since, however, their observations were made on nuclei isolated in aqueous media, it seems probable that their findings only serve to emphasise the high probability of losses of constituents of small

molecular size when aqueous media are employed for nuclear isolation.

PART II.

Methods for the determination of the relative DNA content of single nuclei.

2.1. Objects of the Present Investigation.

Optical methods for the measurement of constituents of single cells have met with severe criticism from several authors, and on a variety of grounds. Of the methods which have been suggested for the estimation of deoxyribonucleic acid in single cell nuclei, we may consider the quantitative use of the Feulgen reaction as suspect in respect of stoichiometry, and possibly also of specificity; methyl green staining in respect of stoichiometry, specificity, and the influence of polymerisation on the degree of staining; ultraviolet absorption measurements in respect of specificity (having regard to possible interference by RNA and by proteins), and of the influence of molecular orientation within the nucleus; while all three methods are open to criticism in regard to the possibility of mechanical, optical or electrical defects in the apparatus employed.

Since the microscopical approach appears to hold out the best, if not the only, prospect of successful chemical study of the individual animal or plant cell, it was considered desirable to attempt a study of how far these criticisms can be justified. All three methods, though depending on quite different physicochemical properties of the molecules of DNA, purport to measure the actual amount of DNA present. If all three techniques could be applied

to the same population of nuclei, gross discrepancies arising from defects of technique should be readily apparent. Furthermore, the considerable amount of published data on the mean DNA content of cell nuclei isolated in bulk from a variety of tissues should provide a reference standard against which the techniques of microspectrophotometry could be assessed.

The present series of experiments, therefore, was chosen with a view to the side-by-side comparison of techniques for microspectrophotometry of individual cell nuclei by the Feulgen, methyl green, and ultraviolet absorption methods. The tissues selected for study were ones for which macrochemical data were readily available; the techniques employed for the quantitative optical measurements were chosen with a view to their easy application to all three methods, and were considerably modified in the course of the experiments. The present section will be devoted to the selection of suitable techniques, and to discussion of alternatives which were discarded after trial.

2.2. Choice of Material for Study.

2.2a. Animal Species.

A number of authors have studied the mean DNA content of nuclei from mammalian sources, and the rat has been the subject of recent extensive studies in this

respect (Thomson, Heagy, Hutchison and Davidson, 1953). In the majority of rat tissues examined, the mean amount of DNA per nucleus has been shown to be reasonably constant, and approximately double the amount found in the sperm head, thus conforming to the hypothesis of Boivin, Vendrely and Vendrely (1948) that somatic nuclei of a range of tissues in a single species all contain the same amount of DNA, the amount being double that found in ova and sperm. However, macrochemical investigations by Thomson, Heagy, Hutchison and Davidson (1953) also showed that certain tissues, notably liver, give higher values for the mean DNA per nucleus than other rat tissues, the differences being apparently greater than could be accounted for by experimental error. With some hopes of resolving this discrepancy, the rat was selected as a suitable species for the present series of photometric studies. The animals used were from the departmental colony, adult male albino rats weighing 250 ± 50 g. being used unless otherwise noted.

In later experiments, a few measurements were made on nuclei of tissue cultures from explants of embryonic chick heart, and on chick embryo liver, with a view to determining whether cell growth and multiplication markedly affect the pattern of nuclear DNA content. The choice of material in this case was determined by the availability of established cultures in this department.

2.2b. Nature of Specimen for Photometry.

The great majority of histological studies on higher animals have employed sections cut from paraffin-embedded portions of fixed tissues, that is to say, tissues in which the proteins have been precipitated, an uncertain proportion of cell constituents removed during fixation and subsequent washing or dehydration, and an equally indeterminate number of substances left behind, possibly in an altered form. Reference to many of the older textbooks of histology will reveal an astonishing variety of recipes for fixative solutions, for each of which some specific advantage has been claimed by its originator. From the point of view of the micro-anatomist or the histopathologist, a fixative which causes severe shrinkage of the protein gels in the specimen may apparently increase the ease of observation of minute detail, but must inevitably produce optical artefacts, not to mention "structures" which did not exist prior to fixation. In cytochemical studies, however, the ideal specimen would be a living cell, provided, of course, that such a specimen were sufficiently static for the necessary measurements to be possible, and provided methods existed for the identification and measurement of cell constituents which did not in themselves involve damage to the cell. At the present time, however, such methods do not exist. Cytochemical methods involving cell staining

with suitable dyes generally require that the cell proteins shall be precipitated, thus providing a static framework within which the location and amount of a constituent may be determined. Methods involving the measurement of absorption of ultraviolet light generally require that the cell shall not exhibit protoplasmic streaming during the period of the measurements; such absorption measurements on the intact cell are also complicated by the difficulty of distinguishing between nuclear and cytoplasmic absorption, since the nucleus is invariably overlaid by cytoplasm, whose thickness may be impossible to determine with sufficient accuracy to make even approximate corrections for its presence. On the other hand, ultraviolet absorption measurements are possible on unfixed cells, and indeed the optical conditions there may be definitely more favourable for accurate quantitative work than in a fixed specimen of the sort regarded as suitable for orthodox histology.

Tissue sections have the considerable advantage that the position of a particular cell or nucleus can be determined, and in tissues where a number of distinct cell types are present, data can be gathered for any desired type. Multinucleate cells can also be recognised. On the other hand, the microtome blade inevitably damages a considerable proportion of the nuclei, more particularly when an attempt is being made to eliminate overlapping by cutting thin

sections. Pollister (Pollister, Swift and Alfert, 1951) has claimed that such sliced nuclei are readily recognisable by careful focussing on to the top surface of each nucleus, but this optimism is not shared by all microscopists. Pollister also suggests that such damaged cells may be recognised by the nucleus giving a lower optical density than one would anticipate, a precaution of even more doubtful validity if one wishes to study the range of variation normally present in intact nuclei.

A serious objection to the use of fixed tissue sections for quantitative cytochemistry arises from the relatively slow penetration of fixatives into even small portions of tissue, so that all cells within a particular section may not have received the same chemical treatment prior to sectioning and staining. (Danielli, 1953).

Finally, one objection which is applicable to the use of sections in quantitative cytochemistry must be mentioned, namely that it is necessary to compare the light transmission of the structure being studied with the light intensity reaching that portion of the specimen. The most practicable means of achieving this is to take a photometer reading through the nearest available clear area of slide, either by moving the specimen aside, or more commonly by arranging for the field to be evenly illuminated, and making a measurement on a clear area adjacent to the

specimen. With tissue sections, this procedure is somewhat cumbersome, unless there are many gaps in the section.

A possible alternative to the use of sections, at least as regards measurements of DNA in single nuclei, is the use of thin films of nuclei which have been isolated, more or less free from cytoplasmic material, by mechanical or osmotic disruption of the cells, followed by differential centrifugation and washing. Such nuclei, of course, cannot be positively identified with respect to cell type, although in tissues such as liver, the size and shape of the nucleus may be sufficiently distinctive to allow a reasonable estimate of the cell type from which it came. Nuclei damaged during the isolation process are usually readily recognisable, except in the case of small irregular nuclei. Suitable isolation procedures can render nuclei resistant to mechanical damage, but cannot guarantee that the batch of nuclei obtained will be a representative sample of those contained in the intact tissue. It is, of course, essential that no significant amount of the material to be measured should be lost from the nuclei during the process of isolation. If this criterion can be satisfied, then isolated nuclei would appear to be the material of choice for ultraviolet absorption studies confined to the nucleus.

Isolation of clean nuclei is not always practicable in every case, some tissues, e.g., lung and muscle,

being difficult in this respect, and in these cases recourse may be had to squash or smear preparations, at least for observations involving the use of selective nuclear stains. Such preparations may also be suitable for ultraviolet observations in those cells where the cytoplasmic contribution to total ultraviolet absorption is negligible, e.g., in thymocytes and spermatozoa.

With these considerations in mind, and having regard to the desirability of comparisons of Feulgen, methyl green, and ultraviolet techniques on the same specimens, it was decided, wherever possible, to use isolated nuclei. In the cases where isolation of nuclei was technically difficult or was unnecessary, smear preparations were used.

2.3. Procedures for Isolation of Nuclei.

The majority of early workers on the chemistry of the cell nucleus realised the desirability of obtaining nuclei free from cytoplasmic material, and Mieschor's original researches (1871) involved the isolation of pus cell nuclei by digestion of the cells with artificial gastric juice, to which the nuclei were relatively resistant. This method has found little application by other workers, since protein contained in the nucleus will also be digested.

Spermatozoa, widely studied on account of their high ratio of nuclear to cytoplasmic material, have been

disintegrated into "heads" and "tails" by dilute acetic acid (Miescher, 1897), and by sonic vibrations (Henle, Henle and Chambers, 1938). The spermatozoon is, however, scarcely representative of the typical mammalian cell, and relatively few recent nuclear studies have been directed to the sperm head. (See Pollister and Mirsky, 1946; Stedman and Stedman, 1943, 1947, 1951.)

Another somewhat specialised cell type from which nuclei can readily be obtained is the nucleated erythrocyte found in birds, fish, reptiles and amphibians, and several methods have been described for freeing such nuclei from cytoplasmic material. All involve rupture of the erythrocyte membrane, e.g., by osmotic means (Ackermann, 1904), or by freezing and thawing (Warburg, 1910). More recent methods involve the use of surface active agents, such as saponin (Dounce and Lan, 1943) or quaternary ammonium salts (Stedman and Stedman, 1951). Whatever agent be used to obtain lysis, the nuclei can be recovered by centrifuging, followed by repeated washing and recentrifugation to free the nuclei from haemoglobin and from red cell "ghosts". Again, the nuclei obtained are not to be regarded as typical somatic nuclei, in that the erythrocyte carries out a limited range of functions, for most of which the nucleus would appear to be unnecessary. Also, the avian erythrocyte nucleus does not ordinarily proceed to mitosis, so

that studies in the cyto-chemistry of mitosis are precluded.

Almost thirty years elapsed after the isolation of erythrocyte nuclei before a method was introduced which has proved to be of general applicability to most cells. Behrens (1932) described a procedure which is still used, involving freezing of the tissue, followed by dehydration of the still frozen cells. The dry tissue is then finely ground, e.g., in a ball mill, and the nuclei are separated from cytoplasmic material by sedimentation in mixtures of non-polar solvents. A suitable mixture is benzene-carbon tetrachloride, the proportions being chosen to give a mixture of lesser specific gravity than the nuclei but greater than that of the cytoplasmic material. Adjustments of specific gravity can be made to suit the needs of particular tissues, and fractions other than nuclei can be obtained if their specific gravity differs from that of other particulate material in the ground tissue.

Among the peculiar merits of the Behrens technique is the retention within the nuclei of the water-soluble components, and the absence of contamination with water-soluble cytoplasmic material. Since fixation by freezing and drying stops enzyme action very rapidly indeed, autolytic phenomena are kept to a minimum, and the chemical state of the nucleus is presumed to be close to that in the living cell (with the exception, of course, of lipids, which

are removed from both nucleus and cytoplasm).

The principal objection to the technique is that it is time-consuming; freeze-drying and grinding even small amounts of tissue require days rather than hours, and unless several sets of equipment are available, only one tissue can be handled at a time.

Other objections are that the nuclear fraction obtained does not consist exclusively of intact nuclei, but may contain both damaged nuclei and nuclei with adherent cytoplasmic material. Also, the organic solvents employed cannot be guaranteed to have no effect on nuclear constituents other than lipids - proteins, for example, may be partially denatured, a relevant objection in enzyme studies.

Despite these drawbacks, the Behrens method has provided valuable information on a wide range of problems. Behrens (1933) used it to obtain haemosiderin granules from spleen, and thyroid colloid (1935), while he also isolated liver nuclei (Behrens, 1939). Bounce, Tishkoff, Barnett and Freer (1950) studied water-soluble nuclear constituents, including proteins and amino-acids, while Allfrey, Stern, Mirsky and Saetren (1952) also employed similar methods in the study of nuclear enzymes. Nuclei isolated in non-aqueous media should prove of value in determining the extent of losses of material from nuclei during other isolation procedures.

Behrens-type nuclei have not been the subject of much quantitative cytochemical study as yet, for several reasons; a) many of the nuclei suffer damage during the grinding process; b) the nucleus isolated in non-aqueous media presents a granular appearance, with sharp refractive index boundaries, leading to extensive light loss by processes other than light absorption, so that photometric measurements may be misleading; c) such a nucleus will contain many water-soluble constituents such as nucleotides and nucleosides, having ultraviolet absorption properties similar to those of the nucleic acids; d) the mounting of such nuclei for ultraviolet investigation presents special difficulties, both as regards selection of a non-absorbing, non-aqueous mounting medium whose refractive index will be suitable for both nucleus and quartz slide, and as regards the preparation of the necessary thin, evenly spread film of single nuclei; e) quantitative staining procedures cannot at present be applied in non-aqueous media.

Methods for the isolation of nuclei in aqueous media have met with considerable success from the mechanical aspect, although considerable misgivings have been expressed regarding the validity of chemical studies on such material. Crossman (1937) isolated muscle nuclei in small numbers by manipulating a fragment of unfixed muscle tissue in 5% citric acid on a microscope slide, such treatment apparently

loosening the attachment of nuclei to the muscle syncitium. An essentially similar process, but on a larger scale, enabled Stoneburg (1939) to obtain fairly large samples of nuclei, both from muscle and from tumours; in this case, however, Stoneburg reverted to the use of proteolytic enzymes to aid in the removal of cytoplasmic material, thereby influencing the protein content of the nuclei also.

Marshak (1941) introduced a simplified method for the bulk isolation of nuclei, again based on the special property of citric acid reported by Crossman, namely, that it apparently disrupts whatever binding material exists between nucleus and cytoplasm. The tissue used was liver, which is readily disintegrated by mechanical means, so that enzymic attack on the cytoplasm was unnecessary. Marshak's method, with modifications, is widely used at the present time; it consists of two main steps. Firstly, the tissue is mechanically disrupted in a dilute solution of citric acid, which serves to free the nuclei from cytoplasmic material, and also appears to exert a toughening effect on the nuclear membrane, so that nuclei treated with citric acid are much more resistant to shearing stress than nuclei isolated in other aqueous media (Mirsky and Ris, 1947). The precise nature of this action is uncertain; it does not apparently involve protein precipitation. Secondly, the components of the disrupted tissue are separated, usually

by centrifugation, re-suspension, and re-centrifugation. For details of procedures, see Dounce (1943) and Mirsky and Pollister (1946).

Since nuclei isolated in citric acid have been made the basis of many chemical studies, it is worth while to consider just what is achieved by this process as ordinarily employed. During the preliminary homogenising of the tissue, commonly performed in a Waring blendor or similar high-speed mixer, the cells are subjected to very high shearing stresses. In friable tissues such as liver and spleen, this results in the breaking-up of the great majority of the cells within two or three minutes, and the proportion of nuclei damaged is relatively small. Fibrous material, however, is much more resistant to shearing stress, and portions of the tissue may therefore survive, relatively intact. Such fragments can usually be removed by straining the homogenate through fine nylon gauze. In tissues which have a high proportion of fibrous material, more prolonged runs of the blendor may be used in an attempt to release more nuclei, though the end-result is commonly that few intact nuclei are obtained, while long strands of connective tissue persist. A compromise must usually be reached between maximum tissue disruption and maximum yield of undamaged nuclei.

A proportion of nuclei is inevitably disrupted

by high-speed mixers, with resulting contamination of the cytoplasmic fractions by nuclear material. The main disadvantage of such loss, as regards studies on the nuclei, is that the final nuclear preparation may not be truly representative of the tissue from which it came. In liver, for example, the relatively small and dense nuclei found in the bile duct cells might be expected to be more resistant to damage than the larger, more loosely packed nuclei of the parenchymal cells.

In the process of isolation of the nuclei from the citric acid homogenate, the commonly used procedure is firstly to centrifuge down all solid material, then resuspend the precipitate in fresh citric acid solution. Centrifugation at low speeds may then precipitate any unbroken cells and small portions of tissue, which are discarded. Centrifugation at rather higher speeds yields a precipitate consisting mainly of nuclei, more or less contaminated with cytoplasmic debris, and repeated resuspension and centrifugation of this precipitate yields, in favourable cases, a preparation of nuclei which on microscopic inspection are seen to be virtually free from recognisable cytoplasmic particles. A number of nuclei, however, not infrequently show tags of adherent cytoplasm, which are not readily removed. In tissues such as liver, where clean nuclei are fairly readily obtained, the extent of such contamination is

usually negligible; in chemical analyses of nuclei in bulk, however, the possible presence of cytoplasmic material must always be borne in mind.

This objection does not arise to any serious degree in the case of isolated nuclei for microphotometric study, since adherent cytoplasm is usually readily recognisable, and such nuclei can easily be rejected.

Aqueous media other than citric acid have been described for the isolation of nuclei from mammalian tissues. Stedman and Stedman (1950) have described in detail the procedure which they have used since 1943, in which tissue pulp is treated with several volumes of 4% acetic acid, and nuclei are subsequently obtained by repeated differential centrifugation in 1% acetic acid. In reply to criticisms by Pollister and Leuchtenberger (1949) that such drastic procedures must reduce the protein content of nuclei, Stedman and Stedman (1951) have quoted experiments in which prolonged contact with 4% acetic acid did not greatly alter the percentages of nucleic acid or of histone in nuclei, as compared with nuclei from the same tissue isolated in saline and not exposed to acetic acid. They also have tested the effect of saline extraction on nuclei isolated in acetic acid, and conclude that "neither 0.85% sodium chloride nor 4% acetic acid removes any appreciable amount of the major components from cell nuclei."

Acetic acid, however, yields nuclei which are intensely granular, and therefore unsuited to photometric study. (For illustration, see Stedman and Stedman, 1950.).

The effect of isolation procedures on the DNA content of the cell nucleus is obviously highly relevant to the present experiments. It is hardly practicable to apply repeated photometric measurements to the same unfixed nucleus after treatment with a variety of agents used in isolation procedures, and therefore in investigating such losses recourse must be had to studies on nuclei in bulk. Dounce (1943) investigated the composition of rat liver nuclei isolated in citric acid at pH values in the range 2.4 to 6.2, and concluded that at pH levels below 3.0, the major portion of the basic protein of the nucleus is lost. Although this does not by itself have any influence on the DNA content of a single nucleus, it will, of course, have the effect of increasing the apparent percentage of DNA in the dried nucleus. Similarly, nuclei isolated at the upper end of the range of pH values obtainable with citric acid (just over 6.0) tend to lose some nucleic acid, but accompanied by the loss of some protein also. From these studies, it would appear that nuclei isolated in citric acid at about pH 4.0 or less are unlikely to have lost any significant amount of nucleic acid, but that nuclei obtained by the citric acid technique are unsuitable for studies

on the protein content of the intact nucleus. Dounce, Tishkoff, Barnett and Freer (1949) in comparing citric acid-isolated nuclei with those obtained by the Behrens technique, found that the percentage of DNA was noticeably lower in the Behrens-type nuclei than in the citric acid sample. This conclusion is consistent with appreciable loss of protein from nuclei during isolation in citric acid.

Early experiments in the present series, made on nuclei isolated in dilute citric acid solution, revealed one serious disadvantage from the microphotometric point of view, namely that citric acid causes aggregation of the chromatin. Fixation of a smear of citric acid-isolated nuclei results in a preparation whose nuclei are optically far from homogeneous. While the microscope image of such a nucleus might be scanned by a photometer or densitometer designed to integrate the light absorption over the entire nucleus, such an instrument was not available to the author. Further, even if accurate integration were possible over the entire image of the nucleus, errors due to light scattering at the interfaces between granules and clear nuclear background would not be corrected. Pollister (1947) has suggested that the photocell used for transmission measurements will itself integrate the absorption of an inhomogeneous nucleus, provided the entire image of the nucleus is projected on to a uniformly sensitive photocathode. That

this is not the case can readily be shown by calculation of the transmission of a sphere or cube with absorbing material uniformly dispersed through it, and that of a body of identical size with the same amount of absorbing material concentrated into, say, 25% of the volume of the body. In such a case a photocell would record an integrated transmission for the inhomogeneous specimen, which would be too high by 20%. In the limiting case, where all absorbing material is concentrated into one infinitely small particle, the photocell will record 100% transmission, i.e., the specimen apparently contains no absorbing material. On the other hand, where the absorbing material is very nearly uniformly distributed through the specimen to be measured, the error is very small, and can be neglected by comparison with other errors inevitably present.

These considerations made it imperative that the nuclear preparations for measurement of transmission or optical density should be as optically homogeneous as possible, and attention was therefore directed to alternative techniques for isolation of nuclei, which would give specimens more nearly approaching the ideal "structureless" nucleus desirable for photometry. Ris and Mirsky (1949) have described a method for preparation of homogeneous nuclei for microspectrophotometry in which the tissue is homogenised in 30% sucrose solution prior to fixation with formalin.

Preparations from rat liver and kidney by this technique were therefore examined; the nuclei in most cases were nearly structureless apart from the nucleoli but there was extensive contamination of the nuclear preparations with cytoplasmic debris which was not removed by repeated differential centrifugation. Treatment of nuclei, isolated in 1% citric acid, with 30% sucrose solution prior to fixation did not affect the granular appearance of the nuclei, indicating that chromatin, once precipitated even by weak acids, is not re-dispersed by concentrated sucrose solutions.

Arnesen, Goldsmith and Dulaney (1949) have described the isolation of nuclei from mouse spleen in a medium containing 0.25M sucrose and 0.008M citric acid. Since the pH of their medium is strongly acid, nucleic acids are unlikely to be lost during the isolation process, and the presence of sucrose in moderate concentration might possibly have the desired effect on the optical properties of the nuclei. Rat liver and kidney nuclei were therefore prepared in the citric acid-sucrose medium, and inspected for homogeneity and freedom from cytoplasmic contamination. The nuclei were found to have a uniform "ground-glass" appearance, similar to that obtained using more concentrated sucrose solutions as described by Ris and Mirsky, but the preparations were extensively contaminated by cytoplasmic

debris, and numerous tags of cytoplasm remained attached to the nuclei. Further preparations were therefore made, in which the initial isolation was performed in citric acid-sucrose medium, but the nuclei obtained were twice washed with 0.01M citric acid without sucrose, prior to the preparation of smears for fixation.

The final product was a clean preparation of nuclei, having the "ground-glass" appearance referred to above, and giving reasonably uniform staining with either Feulgen's method or with methyl green. The detailed technique used for the preparations is described below.

2.3a. Methods employed for isolation of nuclei in the present experiments.

Adult male albino rats, weighing 250 g $\pm$ 50 g. were used, and were fed a stock "rat cake" diet ad libitum (Lever Cattlefoods Ltd.). In experiments on hepatoma tumour nuclei, liver tumours were induced by feeding a diet containing 0.06% p-dimethylaminoazobenzene in a casein-glucose-arachis oil-salt-vitamin mixture (Griffin, Nye, Noda and Luck, 1948). In experiments involving regenerating liver, partial ($^{2}/_{3}$rds) hepatectomy was performed by the method of Higgins and Anderson (1931). The animals were killed 48 hours after hepatectomy.

In all cases, the animals were lightly

anaesthetised with ether, exsanguinated from the cervical vessels, and the appropriate organs (liver, kidneys, pancreas, or small intestine) were removed as rapidly as possible and chilled in ice. Each organ was weighed, and was then homogenised for 2 min. in 4 or 8 volumes of an ice-cold solution containing 0.25 M sucrose and 0.008 M citric acid. The blender used was an M.S.E.-Nelco model, run at maximum speed (approx. 14,000 r.p.m.). The resulting homogenates were strained twice through fine nylon gauze to remove any fibrous strands resistant to homogenisation, and were then centrifuged for 10 min. at 880 g. in a refrigerated centrifuge. The supernatant liquid, consisting mainly of cytoplasmic material, was discarded, while the sediment, consisting of nuclei, unbroken cells, and particulate cytoplasmic material, was re-suspended in 4 vols. of the sucrose-citric acid solution at 0 deg. and was centrifuged at 500 g. for 6 min. The supernatant was again discarded, the sediment being again resuspended in the citric-acid-sucrose medium and re-centrifuged at 500 g. as before, this operation being repeated until the sediment proved on microscopic examination to consist almost entirely of cell nuclei. In most cases, a total of from four to six cycles of sedimentation and re-suspension yielded an acceptably clean preparation of nuclei. This nuclear sediment was then re-suspended and re-centrifuged twice at 500 g. for

six minutes in 0.01 M citric acid solution. The resulting nuclear preparation could be stored in a refrigerator at 4 deg. for not more than 24 hours, and was employed both for direct chemical estimation of DNA and for microspectrophotometric estimations, as described later.

2.3b. Methods employed for chemical estimation of the mean DNA content of rat cell nuclei.

1). Estimation of numbers of nuclei in a preparation. The suspension of nuclei in 0.01 M citric acid was thoroughly shaken, and a sample was quantitatively diluted 100 times in the same medium, using an erythrocyte pipette. The diluted sample was counted in a haemocytometer of the Neubauer type, at least 1000 nuclei being counted for each preparation. Duplicate estimations were performed.

2). Estimation of the amount of DNA in a known number of nuclei. The nuclear suspension was analysed by a modification of the method of Schmidt and Thannhauser (1945). A 5 ml. aliquot of the suspension was placed in a 15 ml. conical centrifuge tube, and to it was added 2.5 ml. of 30% w/v trichloracetic acid at 0 deg. The mixture was stirred vigorously, then stood in an ice bath for 30 min., then centrifuged to separate the precipitated proteins and nucleoproteins. The supernatant was discarded, and the precipitate was re-suspended in 3 ml. of ice-cold 10% w/v

trichloracetic acid, centrifuged, then resuspended and re-centrifuged in 10% trichloracetic acid twice more. The precipitated material was then successively extracted with 5 ml. portions of acetone, ethanol, ethanol-chloroform 2:3 mixture, ethanol-ether 3:1 mixture twice, and ether. The resulting acid-insoluble lipid-free material was dried in air, and was then stirred with 3 ml. of N NaOH solution, lightly stoppered, and placed in an incubator at 37 deg. for 18 hours. Under these conditions, the RNA of the sample is converted to acid-soluble nucleotides, while the DNA, though altered, remains acid-precipitable.

The alkaline digest was cooled in ice, then brought to pH 1 by the addition of ice-cold 50% w/v trichloracetic acid, stirred thoroughly, placed in an ice bath for 30 min., and centrifuged. The supernatant was retained, and to it were added two 3 ml. washings from the precipitated material, using 10% w/v trichloracetic acid. The precipitated DNA and protein was dissolved in a small quantity of N NaOH (usually 1-2 ml.), and the amount of DNA present was estimated by measuring the phosphorus content of aliquots, using the method of Allen (1940). The same method was used for determination of the RNA phosphorus contained in the pooled supernatant and washings.

2.3c. Preparation of tissue culture specimens.

Chick heart fibroblast cultures were obtained from Dr. I. Leslie. They had been grown by the roller-tube technique from implants of embryonic chick heart (Davidson and Leslie, 1951), which had been made on to glass coverslips in contact with the walls of the roller tubes. The cultures were harvested at 120 hours after implantation, the dense central portion of the implant having been removed at 36 hours, in order to provide cultures which were of single-cell thickness and attached to coverslips.

The coverslips, as received, were drained of the serum-embryo extract culture medium, and were immersed in the citric acid-sucrose medium described above for 30 min., rinsed in 0.01 M citric acid to remove the sucrose, drained, immersed in acetic acid-ethanol 1:3 mixture for 30 min., and washed.

2.3d. Smear preparations.

Where the amounts of material available did not permit of isolation of nuclei, as in the case of rat sperm obtained from the cut vas deferens, and chick embryo liver, the available material was first immersed in the citric acid-sucrose solution, then smeared directly on one end of a microscope slide, fixed in acetic acid-ethanol as above, and washed.

2.4. Methods for Fixation, Staining and Mounting of Nuclei.

2.4a. Attachment and Fixation of Isolated Nuclei.

A preparation of isolated nuclei, suspended in an aqueous medium, is unsuitable for direct examination and cytochemical measurement, since the free nuclei are highly mobile and even in thin films are difficult to photograph without blurring. For ultraviolet microphotography, where some time must elapse between the location of a suitable field and its successful focussing, it is necessary that the nuclei should in some way be attached to the microscope slide. For stained preparations, and particularly for Feulgen staining, which involves acid hydrolysis of the specimen at an elevated temperature, the attachment of the nuclei to the slide must be even more secure. Attachment of the nuclei by means of protein films such as egg albumen is unsatisfactory for ultraviolet work since the protein has appreciable ultraviolet absorption properties, while during hydrolysis prior to Feulgen staining, even fixed protein films are liable to become detached from the slide.

Preliminary experiments were made in which wet films of nuclei were formed on the slides by the usual methods applicable to blood films, and were subjected to a variety of common fixatives including Carnoy's acetic-alcohol mixture, formol-saline, mercuric chloride, Zenker's fluid. It was invariably found that the fixative removed all

nuclei from the slide. Films were also prepared and allowed to dry in air at laboratory temperature, and were then submitted to the acid hydrolysis required for Feulgen staining. Once again, virtually all the nuclei were removed from the slides. A combination of air-drying and fixation with either acetic alcohol or formol saline proved effective in securing the nuclei to the slides, and contrary to expectation, the nuclei when examined microscopically did not show crenation or other gross distortion resulting from air-drying. Subsequent preparations were therefore spread on slides in a thin film, allowed to dry at about 18 deg., and thereafter treated with fixatives.

The question of which fixative agent is most suitable for microspectrophotometric studies cannot be answered simply. In the Feulgen reaction particularly, the intensity of the staining reaction varies with the nature of the fixative, as well as with the duration of fixation and with the size of the block of material which is immersed in the fixative solution (Swift, 1953, 1955). It appears possible that rapidly penetrating fixatives may, in whole tissue blocks, prevent protein loss by autolysis, a process which appears to exert some influence on the final intensity of Feulgen staining (Lhotka and Davenport, 1951). Fixatives containing formalin must be regarded with suspicion for Feulgen staining, since residual aldehydes might

materially increase the staining intensity, and even prolonged washing may not be fully effective in getting rid of surplus fixative. A fuller discussion of the effects of fixatives may be found in papers by Hillary (1939), Molovidov (1936), and Swift (1955). For the present experiments, preliminary trials showed that fixation with acetic-alcohol (Carnoy's fluid) was equally suitable for Feulgen, methyl green, and ultraviolet work, and accordingly this fixative was employed for all preparations used in the present series of experiments.

The specimens (smears of isolated nuclei dried on slides, spermatozoa, smears of chick embryo liver, and chick heart fibroblast cultures) were immersed in acetic acid-ethanol (1:3 mixture) at room temperature for 30 minutes, removed, drained, and washed in running tap water for 2 hours.

In view of the possibility that minor variations in fixation and staining techniques might lead to differences in stain intensity on different slides, thereby invalidating comparisons between specimens, it was decided to employ on every slide a control sample of nuclei which could be used as a basis for comparison between the different specimens. For this purpose rat kidney nuclei were chosen, since their mean DNA content per nucleus is reasonably constant (Thomson, Heagy, Hutchison and Davidson, 1953), and since

early photometric experiments in the present series showed that within a population of isolated kidney nuclei the individual variation in DNA content is small. A typical specimen for staining, therefore, consisted of a slide having on one end a smear of rat kidney nuclei and on the other a smear of nuclei, spermatozoa, etc. whose DNA content was to be determined. Since both samples were fixed, stained and mounted simultaneously, variations due to minor changes in these processes should affect both sample and control to an equal extent.

2.4b. Staining and Mounting of Feulgen Preparations.

The Feulgen reagent was prepared as described by Coleman (1938), his procedure being essentially an improved version of that described by de Tomasi (1936).

> 1 g. basic fuchsin (B.D.H., Ltd.) was dissolved in 200 ml. boiling water, then cooled to 50 deg. and filtered. To the filtrate were added 2 g. potassium metabisulphite and 10 ml. N hydrochloric acid, and the flask was stoppered. After 24 hours, the residual brown colour was removed by shaking the solution vigorously for 1 min. with 0.5 g. animal charcoal, followed by rapid filtration. The resulting colourless clear solution was stored in a tightly stoppered flask in the refrigerator. Batches of the stain were discarded after use.

The resulting leucofuchsin solution remained stable when stored in a refrigerator for up to six months. Any batches showing a pink or brown colour were discarded.

Acid metabisulphite rinsing solutions were pre-

pared as described by de Tomasi (1936).

> 5 ml. of N hydrochloric acid and 5 ml. of 10% (w/v) potassium metabisulphite were mixed and made up to 100 ml. with distilled water.

The acid metabisulphite solutions were freshly prepared weekly, and were stored in stoppered bottles at room temperature.

The duration of acid hydrolysis of the preparations prior to Feulgen staining was chosen to represent a compromise between over-hydrolysis, with resulting loss of Feulgen stainability, and under-hydrolysis, leading to insufficient uncovering of aldehyde groupings. In view of the use of a control specimen on every slide, the precise duration of hydrolysis should be less critical than when no such comparison standard is employed. Hydrolysis in N hydrochloric acid for six minutes at 60 deg. was found to give satisfactory staining, and was used in all Feulgen preparations. (For a discussion of the effects of varying conditions of hydrolysis, see Swift (1955)). The detailed procedure for Feulgen preparations was as follows:-

The fixed and washed slides were first immersed in N HCl at room temperature, and were then transferred to a staining jar containing N HCl at 60 deg., and maintained at that temperature by a thermostatically controlled water bath. After six minutes in the hot HCl bath, with occasional gentle agitation, the slides were removed with

stainless forceps, rinsed momentarily in cold N HCl, then in cold tap water, and drained. The slides were then transferred to the leucofuchsin solution, contained in covered staining jars, and were stained for two hours at room temperature (18 deg. approx.) without agitation. The slides were then removed, drained on to blotting paper, and immediately transferred to the first acid-metabisulphite rinsing bath. After thirty minutes in the first bath, at room temperature, they were drained and transferred to the second bath for a further thirty minutes. Finally, the slides were washed for five minutes in running tap water, allowed to drain and dry at room temperature, and were mounted in "D.P.X. Neutral Medium".

Where coverslips carrying chick embryo heart fibroblast cultures were to be stained, slides carrying the control smears of chick embryo liver cells were hydrolysed, stained and rinsed simultaneously in the same jars, the tissue culture coverslips being ultimately mounted on the unused halves of the slides carrying the embryo liver smears.

2.4c. Staining and Mounting of Methyl Green Preparations.

It is noteworthy that at least two distinct dyestuffs are marketed for histological purposes under the name of "Methyl Green". One, sold as "Gurr 150", "Gurr 3524", and "National Aniline Corpn. Cert. 26, C.I. 685", is stated to consist of either heptamethyl pararosaniline or

ethylated hexamethyl pararosaniline or a mixture of both in unspecified proportions, and is always contaminated with a varying amount of methyl violet, which, however, is readily removed by extracting the aqueous solution of the dye with chloroform, the extraction process being repeated until the chloroform layer is colourless. The staining properties of methyl green are identical irrespective of whether the ethylated hexamethyl- or heptamethyl- dye is employed (Kurnick and Foster, 1950).

The second "Methyl Green", marketed under the names "Methyl Green B.D.H. Standard Stain", "Gurr 1045", "Gurr 748", "Flatters and Garnett Methyl Green", "Flatters and Garnett 4260", is said by one manufacturer (Gurr) to be a different dyestuff, not chemically related to the pararosaniline series, but possessing allegedly identical staining properties. Preliminary experiments, however, showed marked differences in the properties of the two dyestuffs, as follows:-

	"Methyl Green Gurr 150"	"Methyl Green B.D.H. Standard Stain"
Solubility in water	Freely soluble	Freely soluble
" " $CHCl_3$	Insoluble	Soluble
Contaminant	Methyl violet	None detected
Wavelength of absorptn. maximum (pH 4.1)	630 mu	618 mu
Staining properties (Tert. butanol differentiation)	Only nuclei stain	Nuclei and cytoplasm both stain

As has already been indicated, the majority of published reports on quantitative methyl green staining have come from Kurnick and his co-workers, and accordingly a sample of the methyl green dyestuff used by him was requested. The material sent by Dr. Kurnick, labelled "National Aniline Corpn. C.I. 685, Cert. No.26", was dissolved in 0.2 M acetic buffer, pH 4.1, to form a 0.25% solution of the crude dye. The solution was then repeatedly extracted with small volumes of chloroform, till no further violet colour could be extracted (approximately 10-15 extractions). A similar solution of "Methyl Green Gurr 150" was prepared and freed from methyl violet, and the absorption spectra of the two solutions were compared, over the range 400-700 mu, using a Unicam Model S.P. 500 spectrophotometer. Figure 2 shows the absorption spectra

Figure 2.

Absorption spectra of solutions of methyl green. (0.25% solutions of the impure dyestuffs in 0.2 M acetate buffer, pH 4.1, extracted with chloroform till the extracts were colourless.)

X------X Methyl green (Gurr, batch No.150)

O------O Methyl green (National Aniline Corpn., C.I. 685).

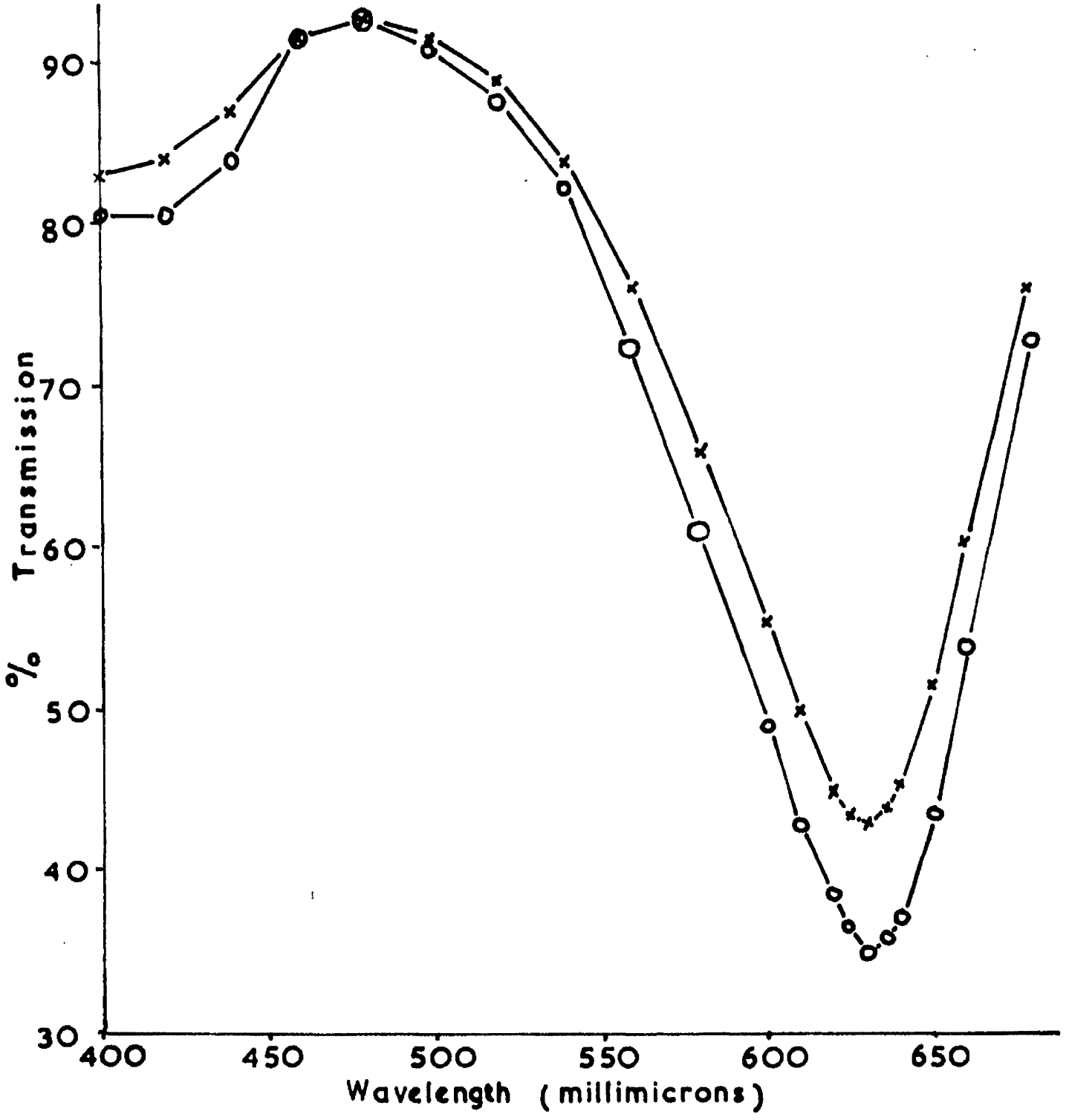
90
80
70
60
50
40
30
% Transmission
400
450
500
550
600
650
Wavelength (millimicrons)

FIGURE 2.

of the two solutions to be identical except for slightly lower dye concentration in the "Gurr 150" batch. Similar spectra were obtained from a batch marked "Gurr 3524", and accordingly all methyl green experiments were performed using either "Gurr 150" or "Gurr 3524" dye.

The methyl green staining procedure initially adopted was exactly as described by Kurnick (1950a):-

Slides, prepared as in Section 2.4a, were placed in 0.1 N HCl for 5 min. at room temperature, and were then transferred to 0.2 M acetic buffer, pH 4.1, for 10 min. at room temperature. The slides were then transferred to the dye solution described above, and allowed to stain overnight at 3-4 deg. in a refrigerator. The slides were then rinsed twice, for 10 min. each time, in 0.05 M acetate buffer, pH 4.1, and mounted in buffer.

The resulting preparations showed no detectable green staining, while several repeat experiments failed to give any different result. Accordingly, attempts were made to repeat in detail the exact fixation and staining procedures used by Kurnick (1950a), using smears of fresh rat liver, teased in 30% sucrose, fixed overnight in 4% formaldehyde, carefully neutralised as described by Kurnick. The resulting preparation, after staining, yielded nuclei having virtually no detectable stain. Repeat experiments, using the "National Aniline Corpn." batch of methyl green

supplied by Kurnick, again gave no detectable staining. The procedure described by Kurnick was therefore abandoned.

An alternative methyl green staining procedure is given by Pollister (1950):- Dry powdered methyl green stain is extracted with chloroform till the extracts are colourless. A 1% solution, in distilled water, of the purified stain is diluted with five volumes of the following mixture:-

phenol	1 g.
glycerol	40 ml.
ethanol	50 ml.
water	200 ml.

Fixed and washed slides are stained in this solution for 15 min. at room temperature, rapidly rinsed in ice-cold water, and differentiated overnight in tertiary butanol, being finally rinsed in xylol and mounted in a neutral medium.

Preliminary trials of the Pollister procedure for methyl green staining gave nuclei whose colour was greenish-purple, while cytoplasm stained pale purple. It appeared probable, therefore, that extraction of the dry stain with $CHCl_3$ had failed to remove all methyl violet. Accordingly, the aqueous solution of the stain was repeatedly shaken with chloroform till the extracts were colourless, and was thereafter diluted with the glycerol/phenol/ethanol/water mixture as before.

Preparations of isolated nuclei, prepared as in Section 2.4a, and stained by this modification of the Pollister (1950) method, consisted of nuclei stained a uniform pale green colour. Nucleoli, where detectable, were unstained, and any cytoplasmic material present was also unstained. The preparations appeared suitable for photometric measurements, and were photographed immediately to obviate any changes due to fading of the methyl green stain (Kurnick, 1950). Using DPX neutral mounting medium, however, no fading of the preparations was detected over periods of up to three months.

2.4d. Preparations for Ultraviolet Microscopy - Ribonuclease Treatment and Mounting Procedures.

A number of preparations for ultraviolet microspectrophotometry were treated with ribonuclease prior to mounting, in order to eliminate the influence of nuclear ribonucleic acid on the total absorption of ultraviolet light by the isolated nuclei. The ribonuclease employed was a crystalline product, prepared according to McDonald's (1948) modification of the procedure described by Kunitz (1940). The preparation had been shown to be free from proteolytic activity*.

Quartz slides, carrying preparations of isolated nuclei, and fixed and washed as described in Section 2.4a,

*The sample of ribonuclease employed was made available to me through the courtesy of Professor J.N. Davidson.

were placed for 1 hr. in a solution containing 0.1 mg. ribonuclease per ml. of 0.02 M citrate buffer, pH 6.75, at 37 deg. They were then rinsed for 5 min. in the same buffer without ribonuclease, dried in air, and mounted in 85% glycerol-water mixture. The quartz coverslips were sealed in position with molten paraffin wax, and slides were photographed within three hours of mounting.

Control preparations were similarly treated except for the omission of ribonuclease from the buffer, and were mounted in glycerol-water as before.

(In a few instances, difficulty was encountered in keeping nuclear preparations for ultraviolet study free from bacterial or fungal contamination. The difficulty was overcome by storing the fixed smears in a desiccator, mounting in glycerol-water being delayed until the specimens were actually required.)

2.5. Optical and Photometric Methods.

2.5a. Optical Apparatus for Microspectrophotometry.

In view of the intended comparison of visible-light and ultraviolet-light methods for the determination of the DNA content of single nuclei, the entire photometric instrument was required to be adaptable for work in both the visible and U-V regions of the spectrum, and to have available the necessary range of light sources to give

nearly monochromatic light at each wavelength at which measurements were required.

The available ultraviolet microscope was a Type M8001 instrument (Cooke, Troughton and Simms, Ltd., York). Plates 1 and 2 show the general features of the apparatus, which is constructed on a heavy base casting mounted on a granolithic slab, which in turn is supported on a masonry pillar forming part of the structure of the building. No difficulties from vibration were experienced, the apparatus being in this respect markedly superior to most photomicrographic equipment.

The casting carries, on kinematic mountings, the microscope body itself (Plate 2), which in principle corresponds to the familiar refracting microscope. It is, however, designed initially for horizontal operation, and is considerably more massive and rigid than the orthodox general-purpose microscope. The microscope tube is of normal length, and takes standard-size eyepieces and objectives, the latter, however, being carried in special kinematic mountings which can be readily interchanged and replaced with a reasonable degree of precision, both as regards focus and centering. The fine-focussing adjustment consists of a calibrated drum coupled to the microscope tube through a gear-train with minimal backlash, the drum being calibrated over a wide range in steps of 1/10 micron.

Plate 1.

General features of the Ultraviolet Microscope.

Seen from left to right are:- "Pointolite" lamp for visible light work; optical bench with cadmium spark source and quartz prisms; exhaust tube to remove spark fumes; light screen with shutter; microscope body; plate camera.

Plate 2.

U-V Microscope body; from left to right are:- substage focussing vernier; substage centering screws; phase-contrast slide and centering screws; mechanical stage; kinematic objective mounting; fine-focussing drums; swing-up fluorescent searcher eyepiece.

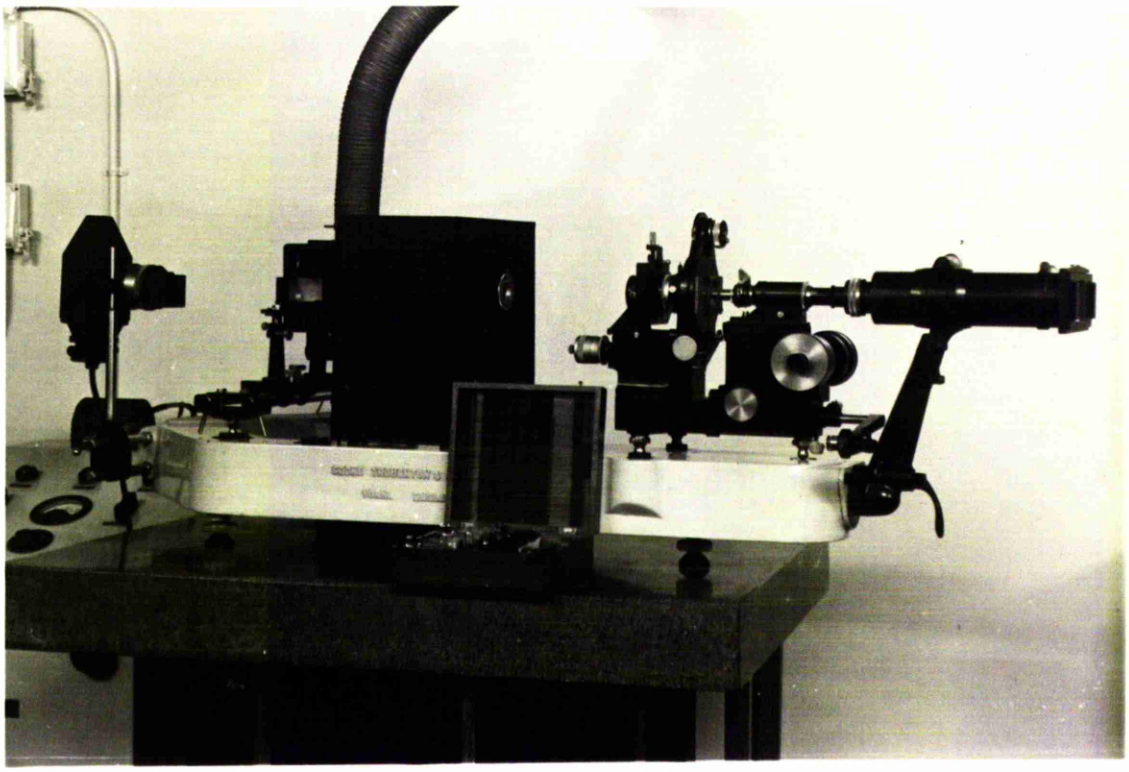

PLATE 1.

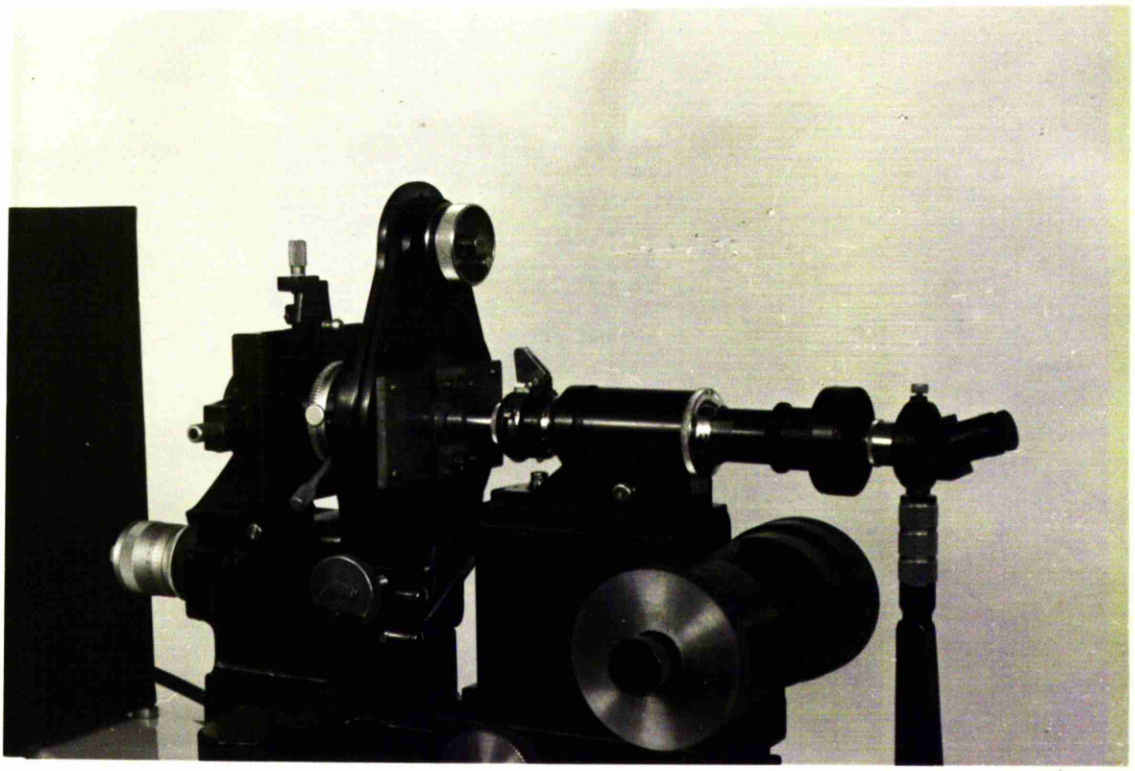

PLATE 2.

With this system, the manufacturers claim high reproducibility of focus settings.

The microscope stage, also on kinematic mountings, is adjustable both horizontally and vertically, as well as being detachable. It is not, however, calibrated on either adjustment. The original slide clips supplied by the manufacturer proved to be insufficiently rigid, as well as being unsuitable for ordinary glass slides, and were replaced by the spring-loaded brass clips and clamping screws visible above and below the objective lens in Plate 2.

The substage mountings are orthodox, and permit of horizontal and vertical movement of the condenser lenses, rapid interchange of condenser mounts, fine substage focussing by calibrated micrometer screw, and introduction of a phase plate for phase contrast work. A normal iris diaphragm is fitted, and there are centering adjustments for the phase plate, independent of the condenser adjustments.

Also mounted on the microscope body is a swing-out fluorescent searcher eyepiece, used for U-V work in conjunction with the quartz ocular, and seen in position in Plate 2.

Mounted on the base casting is a rigid swing-out arm carrying alternative camera bodies, which connect to the microscope through a light-tight metal sleeve. Plate 3 shows the plate camera with ground-glass focussing screen

Plate 3.

U-V Microscope Body, with fluorescent eyepiece swung down, and plate camera with focussing screen in position.

Plate 4.

Microscope body with 35 mm. camera adaptor and Leica camera body.

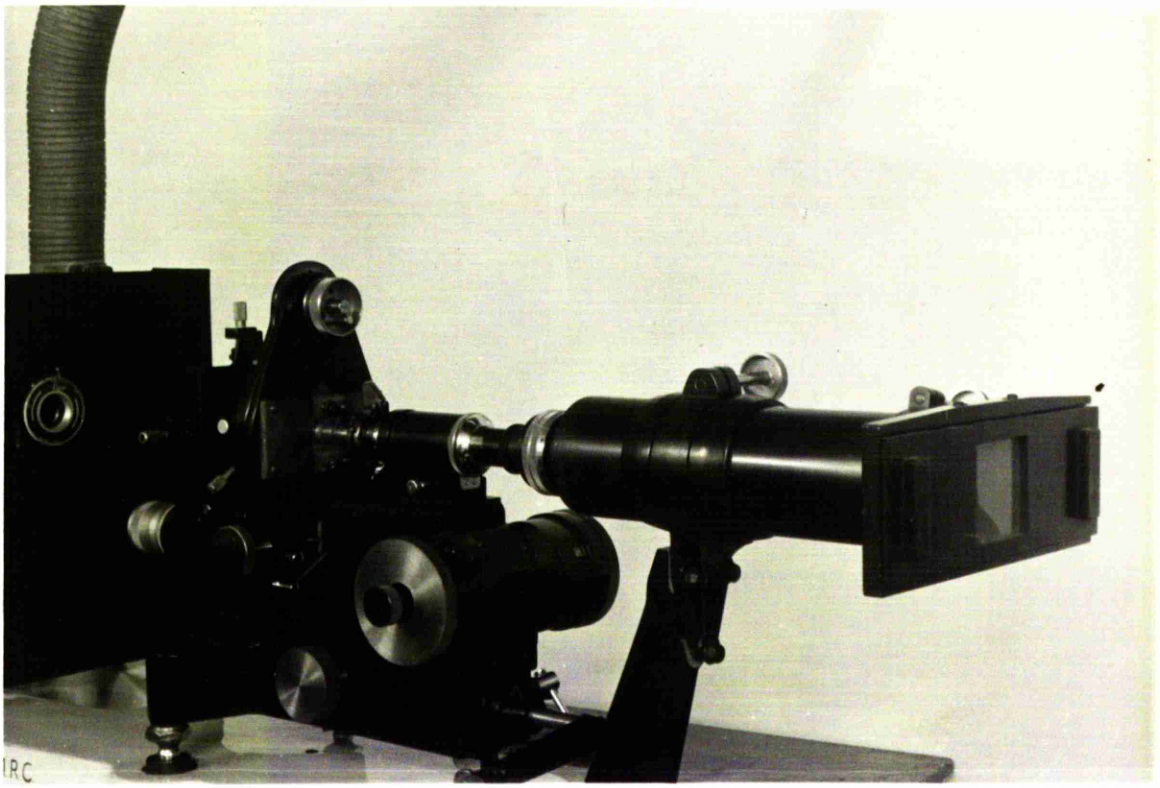

PLATE 3.

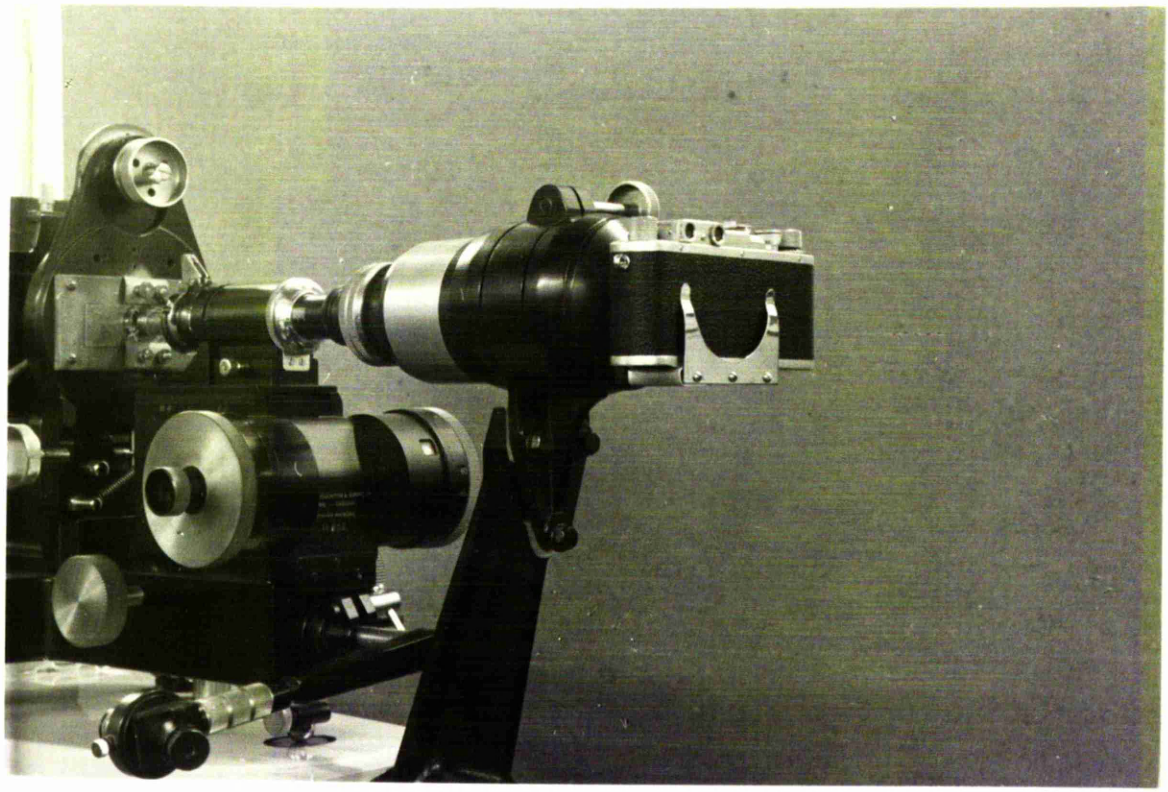

PLATE 4.

in position, while Plate 4 shows the 35 mm. film adapter used in conjunction with a Leica camera body, no lens being used between the microscope ocular and the roll film. Plate 5 shows the camera adapter with the camera body removed, and a focussing screen in position. The screen consists of a sheet of ground glass coated on the surface nearer the microscope with a thin film of anthracene (evaporated from a benzene solution of crude anthracene). This screen was found to be suitable for rough focussing in both visible and ultraviolet light. A metal light-screen is mounted between the microscope and the light sources, and carries a photographic shutter.

The instrument has a number of alternative light sources. For visible light work there is a "Pointolite" tungsten arc, seen on the extreme left in Plate 1, having adjustable condenser lens, iris diaphragm and filter carriers, and mounted on universal supports allowing ready adjustment horizontally and vertically.

For ultraviolet work, there is an optical bench carried on kinematic mounts, and shown separately from the remainder of the instrument in Plates 6 and 7. The main U-V light source is a spark gap between cadmium electrodes, operating at 10 KV, 500 c/sec. alternating current, supplied from a motor alternator and high voltage transformer (not shown). The principal emissions of such a spark are the

Plate 5.

U-V microscope body, with fluorescent focussing screen in place of 35 mm. camera body.

Plate 6.

Light source optical bench; from left to right are:- spark gap between cadmium electrodes (cover removed): quartz condenser lens with iris; prism table with quartz prisms.

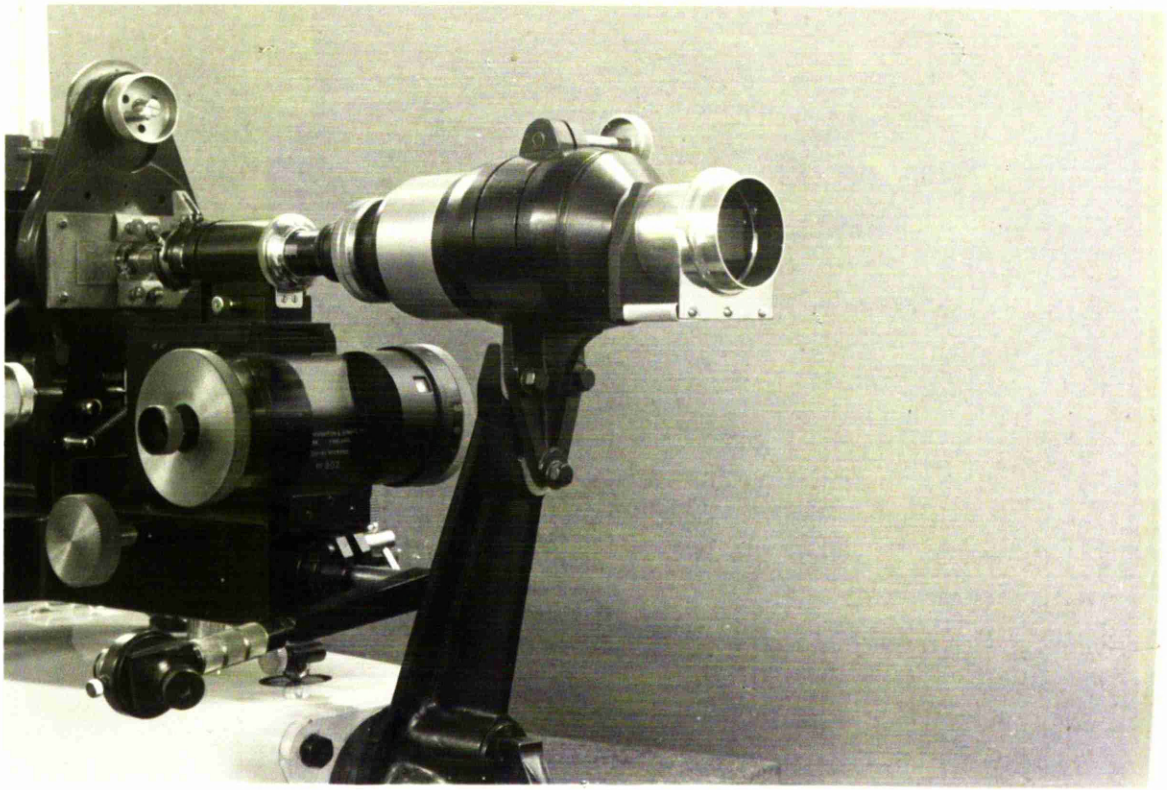

PLATE 5.

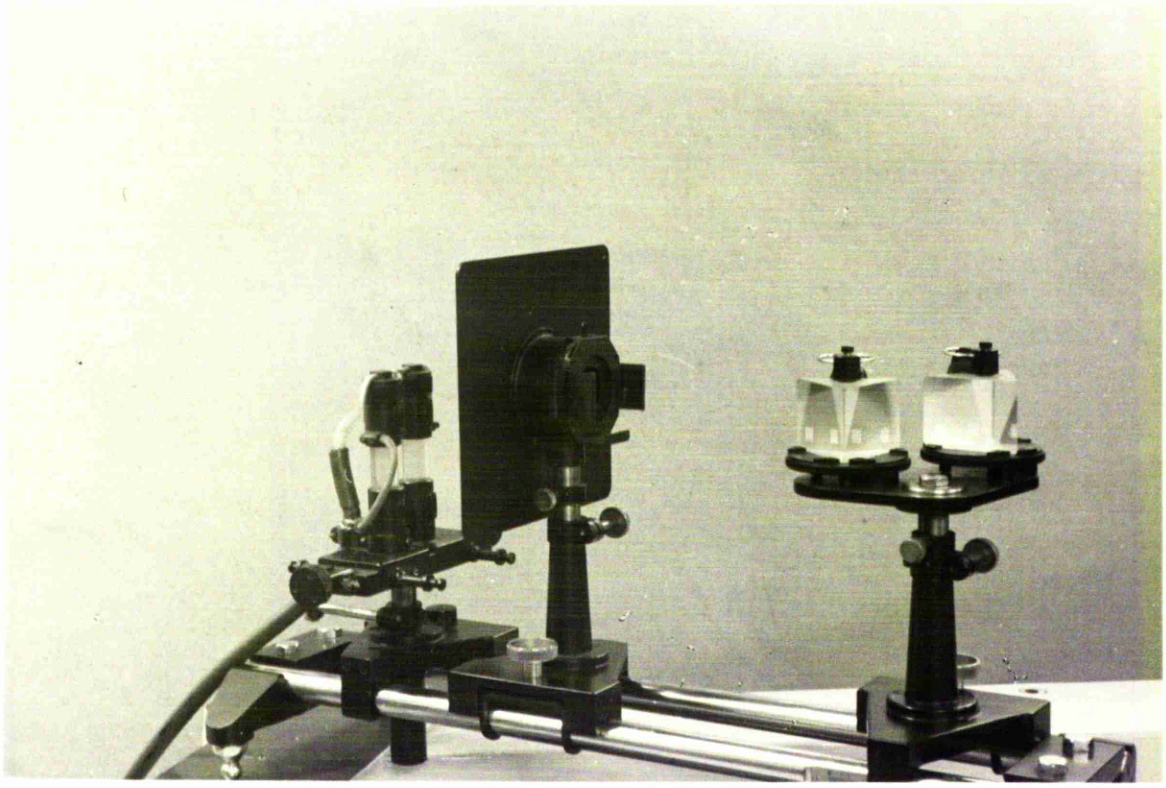

PLATE 6.

2570 A and 2750 A lines, either being made available by means of a double quartz prism system, the entire optical bench being moved on its mountings to project the required line into the microscope optical path. The wavelengths of light in use were checked by a small hand quartz spectroscope (Beck, London), held in the microscope axis.

An alternative U-V light source is the mercury resonance lamp, a cold cathode low pressure mercury discharge lamp in a quartz tube (Plate 7). Operated at 1000 v, 50 c/sec. alternating current, such a lamp emits the major portion of its light energy as the 2536 A line. A single quartz prism serves to isolate this line from other mercury emission lines, which are of relatively low intensity in a lamp operating under these conditions.

It is to be noted that neither of these light sources is inherently stable, the cadmium spark source in particular being liable to very rapid fluctuations in intensity for which no effective remedy exists. Provision is also necessary for removal of cadmium vapour from the spark chamber by an exhaust fan, while interference with other electronic equipment by direct radiation from the arc and by mains-borne interference makes the use of the cadmium spark source undesirable if any alternative can be found. Even total screening of the power supplies, connecting cables, and spark chamber except for the light aperature,

Plate 7.

Light source optical bench, with low-pressure mercury vapour discharge lamp. Note the bench mountings, consisting of a kinematic pivot at the right-hand end, and two steel ball-feet at the left-hand end, resting on a ground steel table. The entire optical bench can rotate about the pivot, permitting selection of light wavelength without altering the alignment of lamp or prism.

Plate 8.

Device for recording a step-wedge on 35 mm. film. The motor-driven 2:1 ratio step-sector revolves in front of a rectangular aperture, behind which is clamped the Leica camera body without lens. The over-all exposure time is controlled by the camera shutter, and adjacent areas of the film receive $\frac{1}{2}$, $\frac{1}{4}$, $1/8$, $1/16$, etc. of this exposure. A tapped transformer (seen on the left) provides an adjustable voltage supply for the electric motor driving the sector, a speed being selected at which stroboscopic effects, from the alternating light source, are not apparent.

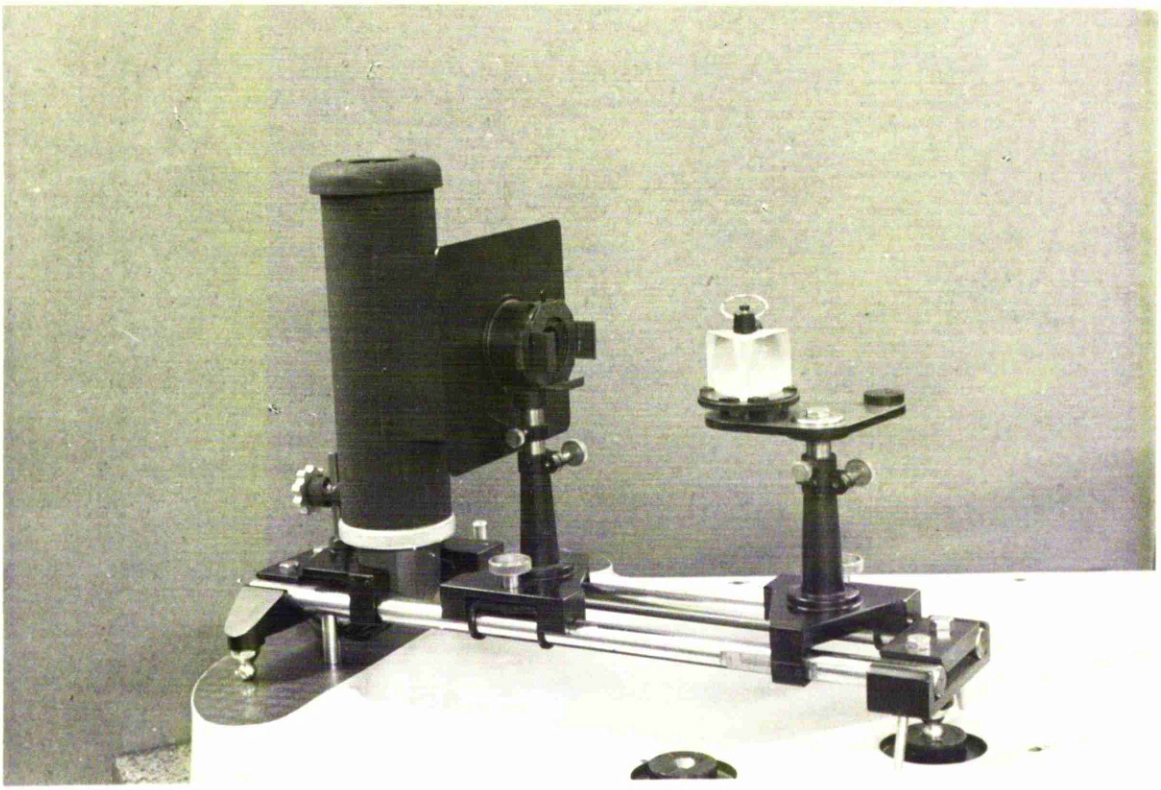

PLATE 7.

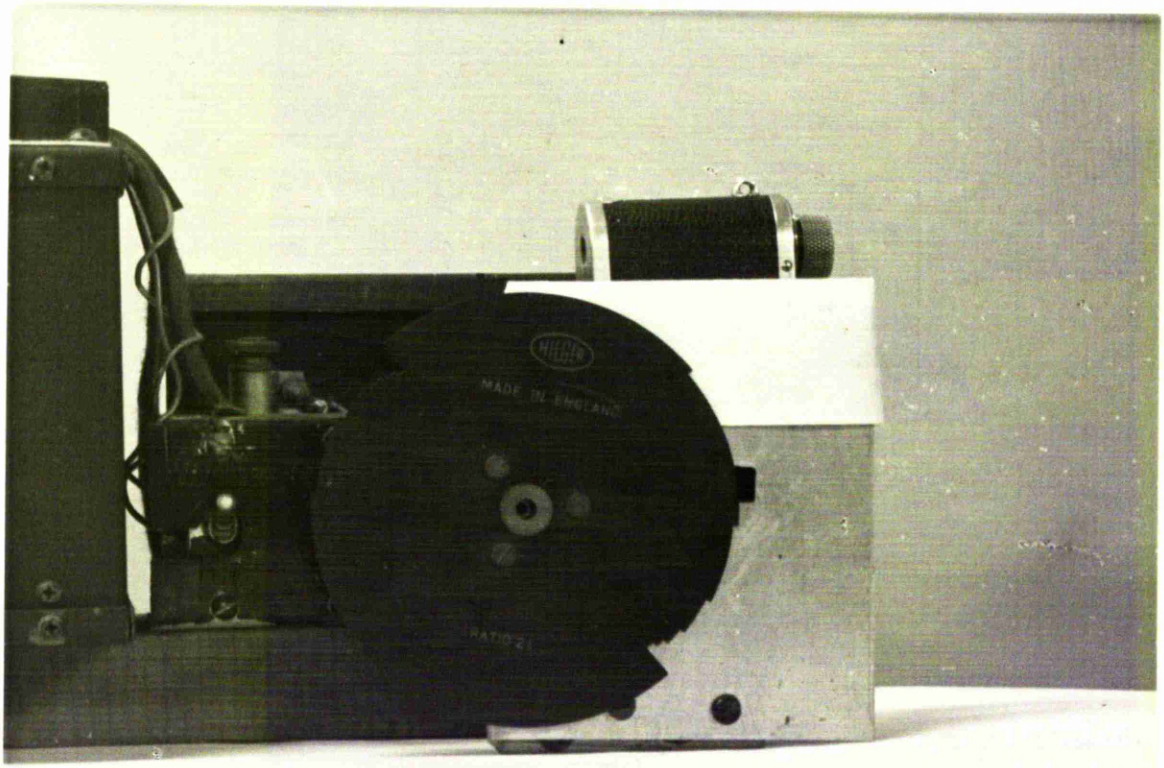

PLATE 8.

did not reduce the interference to acceptable levels, and accordingly the cadmium spark source was rarely used during the present experiments.

A second visible light source was adapted to the instrument, consisting of a 75 w medium pressure mercury arc (Siemens "Sieray") operating at 250 v 50 c/sec. via a ballast choke. When used in conjunction with a "mercury green" colour filter (Hilger), and a single prism, this lamp provides monochromatic light at 5460 A for measurements on Feulgen-stained material.

Alternative optical systems were employed for the three types of mensurements, as follows:-

For Feulgen preparations:

LAMP	Medium pressure Hg arc.	PRISM	One
FILTER	Hilger "mercury green"		λ= 546 mμ
OBJECTIVE	3.75 mm. fluorite apochromat, N.A. 0.95, glycerol immersion		
OCULAR	10x, glass. Ocular-to-film distance 22 cm.		

For methyl green preparations:

LAMP	"Pointolite"	PRISM	None
FILTERS	Ilford type 607 with Chance OR2		λ= 630 mμ Bandwidth for half-intensity = 60 mμ

OBJECTIVE and OCULAR as for Feulgen work.

For U-V measurements of nucleic acids:

LAMP	Quartz low-pressure Hg resonance type.
FILTERS	None λ = 254 mμ PRISM One (quartz)
OBJECTIVE	2 mm. fused quartz, corrected for 257 mμ, N.A. 1.25 glycerol immersion.
OCULAR	10x crystalline quartz.

Normally, the substage condenser was quartz, glycerol immersion, N.A. 1.25, used with 6 mm. diaphragm aperture. (For a few check experiments on the possible influence of glare in the optical system, the substage condenser was removed and replaced by a 4 mm. Leitz apochromat objective (dry)). Extra-thin (1 mm.) slides were used, on account of the close working distance of this lens, and in front of the lamp condenser was placed a metal plate having two circular apertures, 1 mm. diameter and 3 mm. apart. This system will be referred to subsequently as "two-spot" illumination.

In all cases, the microscope field was evenly illuminated on the Kohler principle, i.e., the light source was first focussed by its condenser lens on to the entrance aperture of the substage condenser. The specimen was then brought into focus, and the substage condenser adjusted until the image of the lamp iris diaphragm was central and in focus along with the specimen. The lamp iris diaphragm was then opened until the whole field to be studied was illuminated.

For visible light work, the setting-up and focussing procedures followed normal photomicrographic practice, while for ultraviolet work, the following procedure was used:-

1). The microscope was fitted with a quartz ocular and a phase-contrast visible-light glycerol-immersion objective. With the quartz slide coated with 85% glycerol on both sides and secured on the stage plate, the quartz substage condenser was brought into contact with the glycerol. The image of the "Pointolite" tungsten ball was focussed on to the substage iris diaphragm, which was closed to 2 mm. aperture. The objective lens was racked down till in contact with the glycerol, and the microscope was roughly focussed on the specimen by eye, closing the substage condenser iris further if necessary. The lamp diaphragm was then fully closed, the substage diaphragm opened to 6 mm., and without altering the microscope focus, the substage condenser was racked up until the image of the lamp diaphragm was in focus with the specimen. The substage condenser was then adjusted so that the lamp iris image was central, and the lamp diaphragm was opened until the field was fully illuminated. The substage diaphragm was opened to 15 mm., and the phase plate moved into position. The ocular was replaced by a telescope with cross wires, focussed on the back image plane of the phase contrast objective, and the

substage phase plate was adjusted until the image of its brightly lit ring coincided exactly with the dark ring of the objective. The telescope was then replaced by the ocular, when a bright-field phase-contrast image of the unstained specimen was obtained. The required field for ultraviolet study was selected and accurately focussed.

2). The phase contrast objective was removed and replaced by a parfocal quartz immersion objective, without altering the microscope focus setting. The phase plate was moved out of the light path, and the "Pointolite" lamp was extinguished. The required U-V lamp (normally the 2536 Å mercury resonance lamp) was brought into position, and was focussed on to a uranium glass screen fitted temporarily into the substage condenser mount. The wavelength of light was checked by a hand quartz spectroscope held in the microscope light path, and the quartz prism(s) adjusted if necessary. The fluorescent searcher eyepiece was swung into position, the uranium glass removed from the substage, and the substage condenser focussed and centred to give maximum illumination in the fluorescent eyepiece field. The microscope focus was then adjusted until a roughly focussed shadow image of the specimen was seen. Kohler illumination was then obtained by the same procedure as outlined above for visible light work. The photographic shutter was then closed to minimise irradiation of the specimen.

During the above procedure, considerable difficulty was usually experienced in locating the approximate focus in ultraviolet light. According to the instrument manufacturers, the various objectives were carefully adjusted after manufacture to give parfocality to within 15 microns, and the kinematic mountings of the objective changer were stated to have the required accuracy to permit of reproducibility in focus when the objectives were removed and replaced. In practice, however, much wider variations were normally encountered, and it was rare to be able to locate the specimen immediately on changing to ultraviolet conditions. Focussing was rendered still more difficult by the very low intensity of the image in the fluorescent eyepiece, particularly when working with the mercury resonance lamp, which is of low specific intensity. It was normally necessary for the observer to be fully dark adapted in order to focus the image at all, and when working single-handed it was usually necessary to carry out the visible light manipulations with one eye closed in order to reserve some dark adaptation for the ultraviolet focussing.

3). Final ultraviolet focus was obtained by making a series of photographic exposures of the same field, altering the microscope focus by 1 to 2 microns between each, and selecting the best setting from inspection of the developed negatives. This tedious procedure could best be

performed by using roll film in a 35 mm. camera, a group of about ten exposures on each side of the approximate focus being usually sufficient to secure an acceptable negative. Since exposures were at high magnification and with very small depth of focus, any adjustment of the microscope stage usually necessitated repetition of the focussing procedure.

2.5b. Photometric Methods - Photoelectic.

In all photometric methods applied to single nuclei, it is necessary to compare the intensity of light transmitted by the nucleus with the intensity of light striking the nucleus, or, in practice, with the intensity of light transmitted by a closely adjacent clear area of the microscope slide. Since it is impracticable to measure the light intensities at the object plane of the microscope, all microphotometric methods are based on measurements of light intensity in the image plane of the microscope, and involve the assumption that the distribution of light energy in the image plane corresponds to that on the observer's side of the object plane. The simplest procedure for comparison of two light intensities is to allow the two beams, in succession, to strike a photoelectric cell, and to compare the currents produced by the cell when so illuminated. In photometry of a nucleus it is, of course, necessary to exclude from the photocell all light except that which has

passed through the nucleus. The amount of light energy available is insufficient to actuate a photocell of the self-generating selenium types, while under these conditions even the much more sensitive vacuum or gasfilled cells require several stages of valve amplification before their output is sufficient to operate a sensitive galvenometer. Caspersson (1940) has shown, however, that specially constructed high-insulation photocells can be successfully used without valve amplification if a quartz-fibre Lutz-Edelmann galvanometer is used. Equipment of this character is difficult to construct and maintain, and does not appear to have found favour in other laboratories, at least as regards microphotometric work. Multistage valve amplifiers, though widely used in electrophysiological studies, are undesirable for precise photometry, since elaborate precautions are necessary to provide a reproducible degree of amplification. Where the photocell output current is extremely low, sufficient stability in the first stages of the amplifier may be very difficult to attain, and in particular the use of such equipment in close proximity to a source of interference such as the cadmium spark is unlikely to be satisfactory.

The advent of photocells of the electron multiplier type within the past decade has rendered practicable the measurement of very low light intensities with a minimum

of associated electronic equipment. The glass envelope of such cells, however, limits their use to the visible and near ultraviolet regions, unless a special envelope is fitted at the time of manufacture. In 1950, when the present experiments were begun, no ultraviolet-sensitive electron multiplier photocell was marketed in Great Britain. A single photocell of American origin was available, however, an R.C.A. type IP 28 nine-stage photomultiplier, having a special glass envelope transmitting ultraviolet light of wavelengths greater than 2500A. Although the sensitivity of this photocell is somewhat reduced near the limit of wavelength, i.e., between 2500 and 2600 A, the stated sensitivity of 3 x 10^6 microamps/lumen is more than adequate for photometric work on cell nuclei.

A photometer was constructed, employing this photocell, and following, in general principle, that described by Pollister and Moses (1949). Some adaptation was necessary, however, to suit the horizontal arrangement of the ultraviolet microscope. The general construction of the photometer is shown in Figure 3. In the initial experiments, batteries were used to provide the necessary high voltage supply for the electron multiplier tube, while a modified commercial valve voltmeter was used to measure the photocell output (Photovolt Model 512, Photovolt Corporation, New York). The circuit of the modified instru-

Figure 3.

General arrangements of photoelectric microspectrophotometer constructed for the present series of experiments.

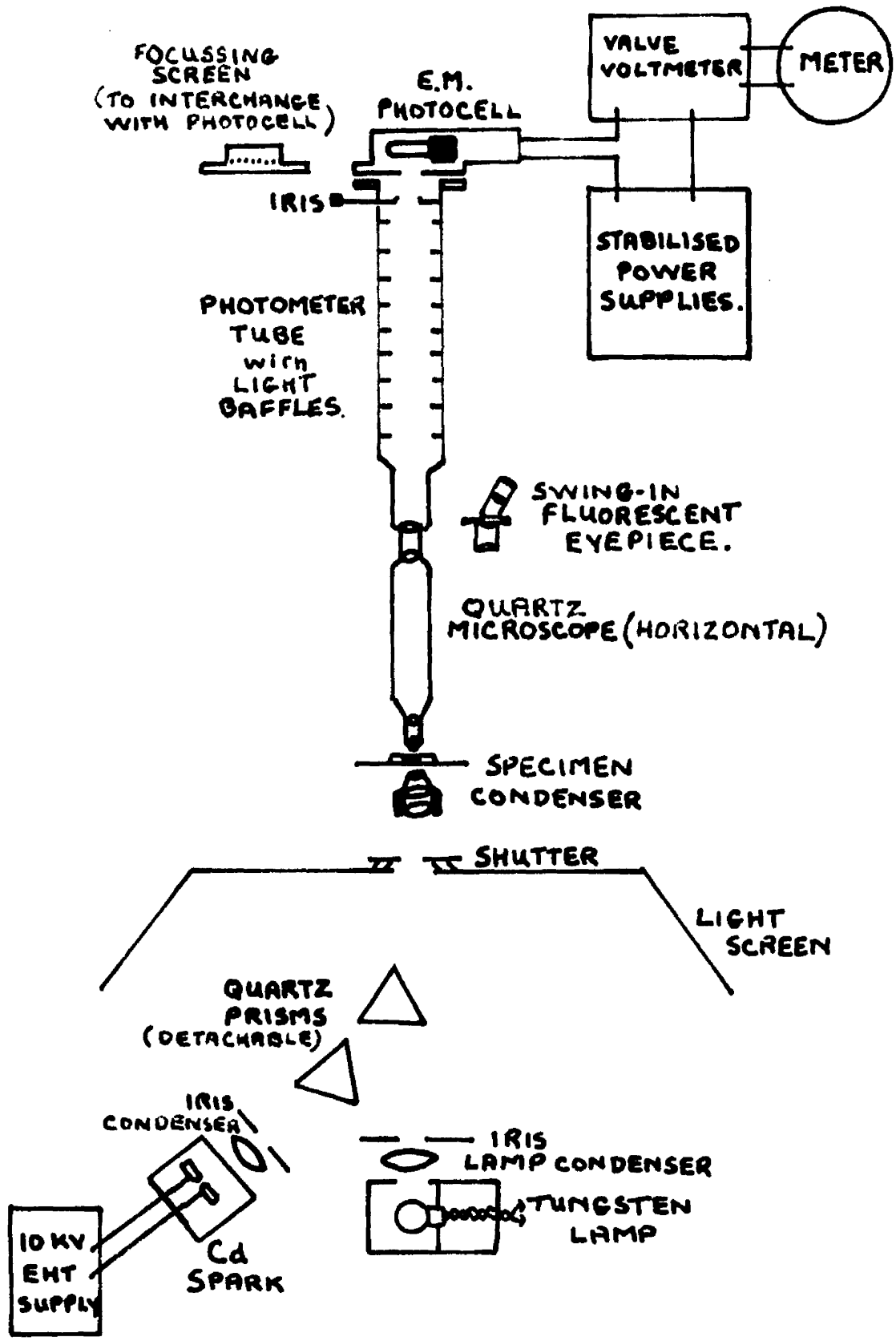
FOCUSSING SCREEN (TO INTERCHANGE WITH PHOTOCELL)
E.M. PHOTOCELL
VALVE VOLTMETER
METER
IRIS
STABILISED POWER SUPPLIES.
PHOTOMETER TUBE with LIGHT BAFFLES.
SWING-IN FLUORESCENT EYEPIECE.
QUARTZ MICROSCOPE (HORIZONTAL)
SPECIMEN CONDENSER
SHUTTER
LIGHT SCREEN
QUARTZ PRISMS (DETACHABLE)
IRIS CONDENSER
IRIS LAMP CONDENSER
TUNGSTEN LAMP
10 KV EHT SUPPLY
Cd SPARK

FIGURE 3.

ment is shown in Figure 4.

Initial measurements, in visible light, showed that the battery operated instrument was not sufficiently stable to give reproducible readings over a period of 1-2 hours, and an attempt was made to construct a mains-operated photometer, again using the I.P. 28 photocell, and in which stability was the primary aim. Figure 5 shows the electronic circuit employed. It consists, firstly, of a stabilised high voltage supply for the electron multiplier tube, using a selected krypton stabiliser as the voltage reference tube, and secondly, of an AC amplifier, diode rectifier, and twin-triode valve voltmeter, designed to take advantage of the alternating light output from a mercury arc when supplied from alternating current mains.

Although the design of the electronic equipment proved to be satisfactory on the test bench, successful use of the instrument for practical microspectrophotometry was not possible, for two reasons:-

a). the ultraviolet light sources were unsuitable. Direct radiated energy from the cadmium spark interfered severely with the valve voltmeter, and efforts at screening were unsuccessful. The mercury resonance lamp proved to be "noisy", i.e., superimposed on its normal 100 c/sec. light output there were random fluctuations of sufficient frequency and amplitude to make accurate photometer readings

Figure 4.

Electronic circuit of modified "Photovolt" battery-operated photometer.

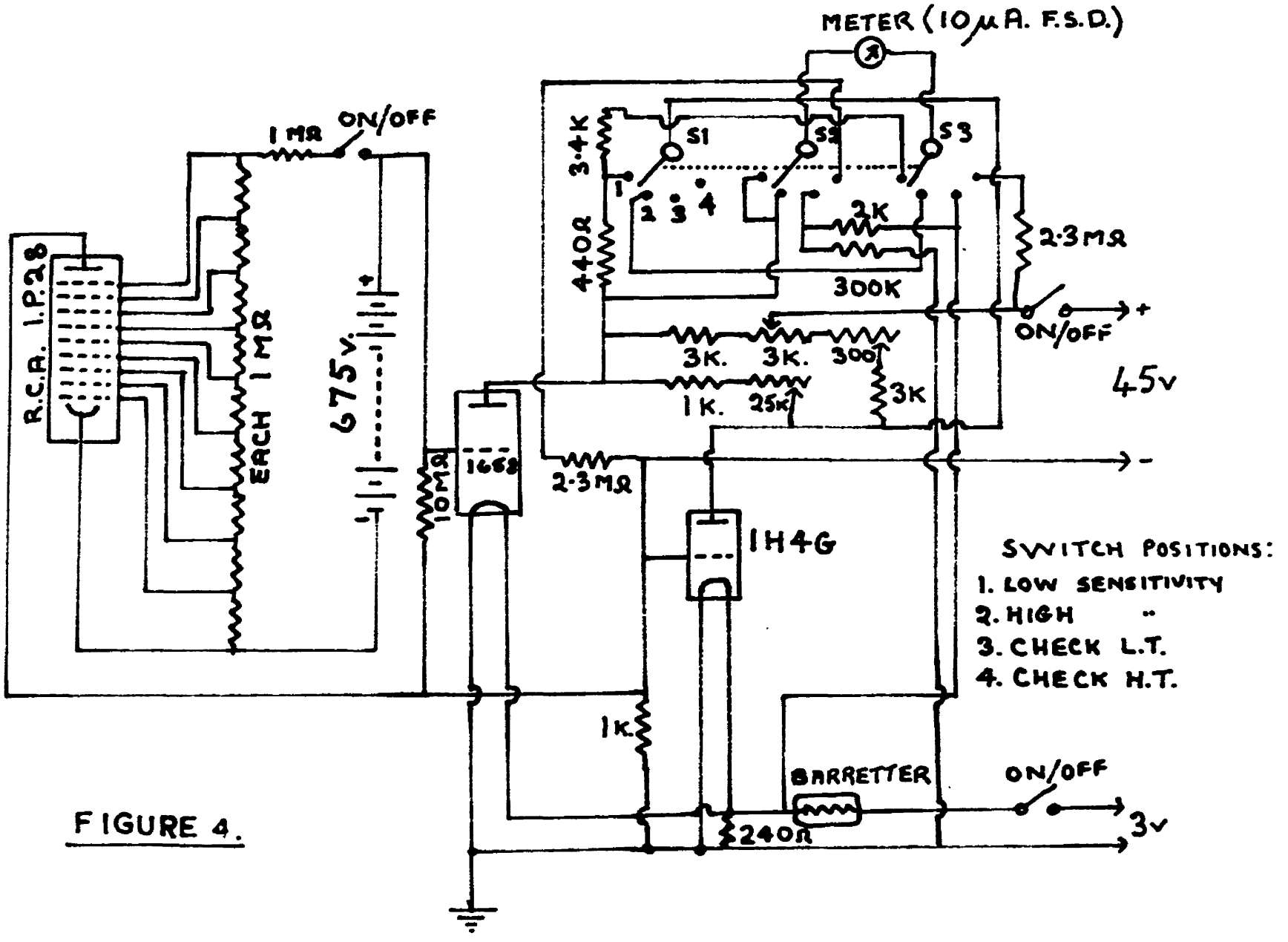
METER (10μA. F.S.D.)
1 MΩ
ON/OFF
R.C.A. 1.P.28
EACH 1 MΩ
67.5v.
10MΩ
166S
2·3MΩ
3·4K
440Ω
S1
S2
S3
1 2 3 4
2K
300K
2·3MΩ
ON/OFF
+
3K. 3K. 300
1K. 25K
3K
45v
-
1H4G
SWITCH POSITIONS:
1. LOW SENSITIVITY
2. HIGH "
3. CHECK L.T.
4. CHECK H.T.
1K.
BARRETTER
ON/OFF
240Ω
3v

FIGURE 4.

Figure 5.

Electronic circuit of mains-operated photometer for visible-light and ultraviolet-light microspectrophotometric measurements.

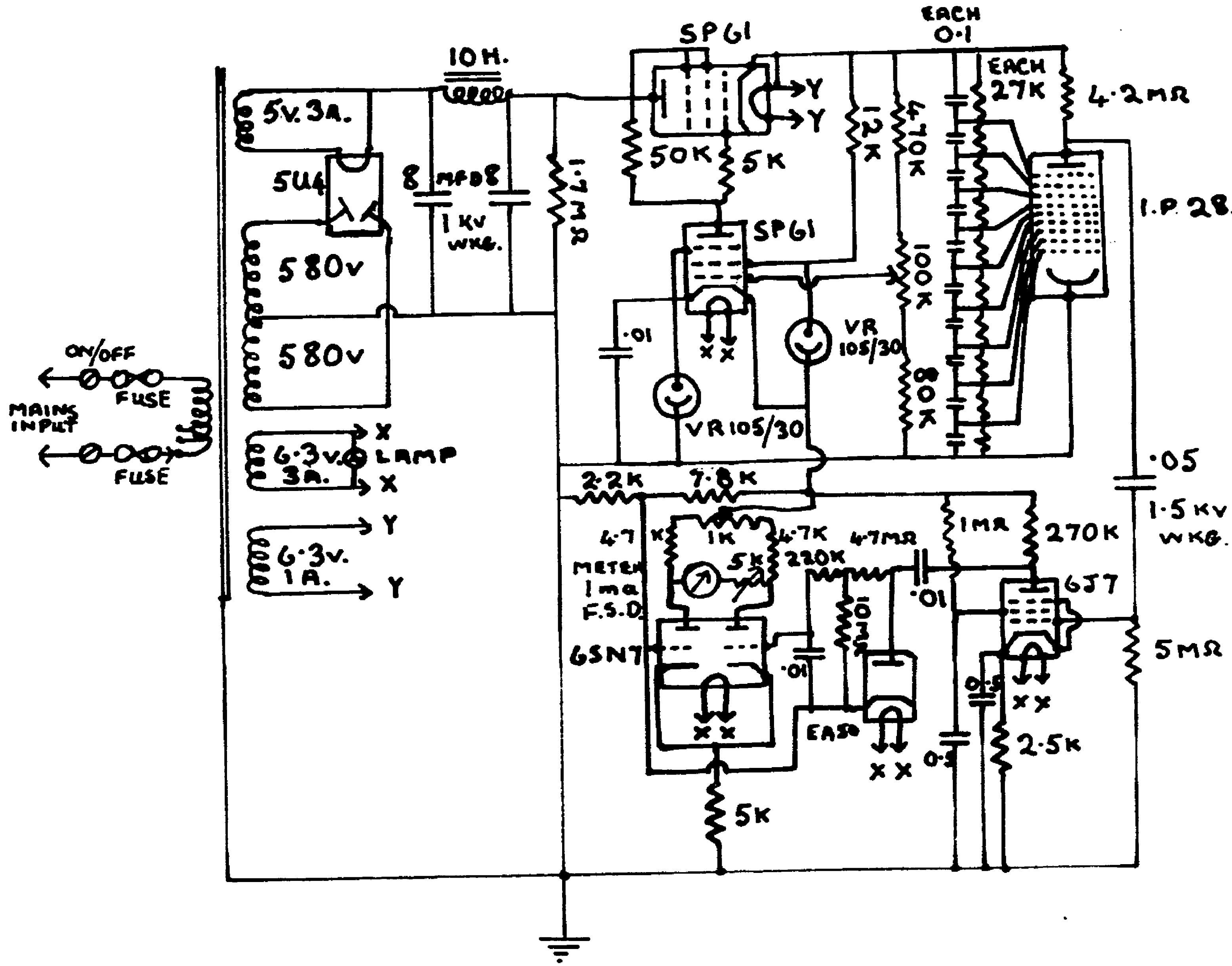
5V. 3A.
5U4
580V
580V
6·3V. 3A.
LAMP
6·3V. 1A.
X
X
Y
Y
ON/OFF
FUSE
FUSE
MAINS INPUT
10H.
8 MFD 8
1 KV WKG.
1·7 MΩ
SP61
SP61
50K
5K
12K
470K
100K
80K
EACH 0·1
EACH 27K
4·2 MΩ
1.P. 28.
VR 105/30
VR105/30
·01
2·2K
7·8K
4·7K
1K
4·7K
5K
METER 1 mA F.S.D.
6SN7
230K
4·7 MΩ
10 MΩ
EA50
·01
1 MΩ
270K
·01
6J7
·05
1·5 KV WKG.
5 MΩ
0·5
0·5
2·5K
5K
XX

FIGURE 5.

impossible. By contrast, stable readings were obtainable when a tungsten filament lamp was employed as the light source;

b). after a period of use, the photomultiplier tube itself became "noisy" and erratic in its response.

Since no alternative photocell was obtainable to replace the unstable one, and also since it was considered essential, for the present experiments, that the photometric system should be equally usable in visible and in ultraviolet light, photoelectric measurements were discontinued in favour of the photographic method to be described in the next section.

Although it was found necessary to abandon photoelectric measuring methods, this is not to be interpreted as a rejection, in principle, of the use of suitable photoelectric apparatus for microspectrophotometric work. The practical difficulties encountered are not by any means insoluble, and photographic methods were adopted on grounds of expediency only. On the basis of a few experiments with photoelectric apparatus, however, it is considered that work in the ultraviolet region on specimens such as nuclei is likely to be exceedingly laborious when photoelectric methods are employed, since locating, focussing and precisely centering each nucleus on the sensitive area of a photocathode is time-consuming and frustrating. It is perhaps

significant that apart from the monumental labours of Caspersson and his school, very few ultraviolet microscopists have reported observations based on photoelectric measurements. The majority of visible-light photometric studies, however, have been made with the aid of photoelectric photometers of the electron-multiplier type.

2.5c. Photometric Methods - Photographic.

Prior to the advent of photoelectric spectrophotometers, it was customary to measure the absorption spectrum of a substance in solution by photographing a line spectrum, such as that of an iron arc, before and after passing through the test solution. Knowing the wavelengths of the various lines, it was possible by comparing the intensities of each line before and after passing through the solution, to draw a curve of the absorption spectrum required. It was, of course, necessary to have both spectra recorded on the same photographic plate, and to have some knowledge of the relationship between exposure and degree of blackening of the photographic emulsion, otherwise the spectrum obtained would be only an approximate estimate. In theory, the manufacturers' characteristic curves for particular emulsions should provide the necessary information, but in practice it is difficult to control development time, temperature, and agitation, as well as effective concentration and pH of the developing bath, and some form of direct

calibration of each plate becomes necessary. For this purpose, two techniques have been widely used, the first being to expose part of the plate to light through a neutral density step-wedge covering a sufficiently wide range of relative exposures. The second, and more reliable procedure, is to expose a portion of the plate behind a rotating disc out of which have been cut stepped sectors, each step subtending, at the centre, an angle related in arithmetic or geometric progression to the angle subtended by the previous step. If each step represents a doubling of the angle of the previous one, then the portion of the plate exposed will, on development, provide a step wedge corresponding to exposures of 1, 2, 4, 8, 16, etc. arbitrary units. If the sector is accurately made in the first instance, then the calibration can be relied on, since modern photographic emulsions are exceedingly uniform; by contrast, the neutral density step wedge, though simpler in use, is liable to error initially and to mechanical and chemical alteration in use. It is also unsuitable for ultraviolet work.

The present experiments were carried out by means of a photographic method which corresponds in principle to the photographic measurement of absorption spectra, except that monochromatic light (or nearly so) was used, giving optical density measurements at one wavelength only. Apart from

the differing light sources and optical components, which have already been described, the procedure was identical for both visible light and ultraviolet light work, and was as follows:-

An appropriate field of nuclei was centred in the microscope field and was carefully focussed, special care being also taken to ensure even illumination of the field on the Kohler Principle. A photographic exposure was made on to 35 mm. roll film, the exposure time being chosen to give a well-exposed negative, i.e., one in which, after standard development, the major part of the tone range of the negative fell within the linear portion of the exposure/density curve for the emulsion in use. The actual exposure time for each experiment was found by trial-and-error exposures on a roll of the same film. A series of fields of the same batch of nuclei was photographed on a single roll of film, as also was a series of fields of the control sample of rat kidney nuclei, referred to in Section 2.4a. The camera was then detached from the microscope, and the latter was removed from its mountings on the base casting of the instrument. The camera was placed, still without lens, behind the 2:1 ratio rotating stop-sector shown in Plate 8, the device being then positioned in the light path which formerly illuminated the microscope. The step sector was rotated at high speed by an electric motor, and the

camera shutter was opened for a suitable period (again determined by trial-and-error exposures). Care was necessary in this operation to ensure that stroboscopic effects did not arise, otherwise the accuracy of the sector exposures might have been invalidated. For this reason a synchronous mains-driven motor is unsuitable, the sector speed being almost certain to coincide with the alternating light output of a mains-operated discharge lamp, but a small series-wound motor with speed control was found to be satisfactory.

The photographic film was then developed, fixed, washed and dried, and ordinarily provided about 20 fields of the nuclei under study, a similar series of photographs of standard rat kidney nuclei, and a calibrating step-wedge. The individual nuclei were then numbered in ink on the film, rejecting all those which were overlapped, out of focus, or obviously damaged. The procedure for density measurements on the negatives is described in Section 2.5d.

The 35 mm. film format (exposures 24 x 36 mm.) was chosen, in preference to plates or larger film sizes, on account of the large number of exposures which can be recorded on a single roll of film for simultaneous development (up to 40 exposures can be accommodated in a standard film cartridge). A Leica camera was used on account of its detachable lens, consistently reliable focal plane shutter

with the necessary range of slow speeds, and availability of scratch-free reloadable cassettes. Other miniature cameras with similar characteristics would have been equally satisfactory.

The film used was a medium speed fine-grain panchromatic emulsion, Ilford type F.P.3. For ultraviolet work there is no advantage to be gained by using high speed orthochromatic or panchromatic emulsions, since their extra speed is only obtained in those parts of the visible spectrum to which they are specially sensitised. Further, although they have excellent exposure latitude, they are somewhat coarse-grained for high resolution work. At the other extreme come the ultra-fine-grain high-contrast slow emulsions, such as Kodak "Microfile", whose characteristics are quite unsuited to microspectrophotometry, their tone range being much too short. F.P.3 represents a reasonable compromise in resolution, tone gradation, and emulsion speed.

Development was in a standard metol-hydroquinone-borax fine-grain developer (Kodak formula D.76), for 10 minutes at 18 deg., using a spiral developing tank, the reel of which was agitated for 5 seconds in every minute during development. No difficulty was experienced due to uneven development, identical step-wedge densities being found when the step-sector exposures were repeated at various points along a film roll. A positive print of a

Plate 9.

Positive print made from a step wedge obtained by the device shown in Plate 8. The numbers indicate the relative exposures received by the corresponding areas of the negative.

Plate 10.

Rat liver nuclei, Feulgen stained, after isolation in citric acid medium. (Compare with Plate 11.)

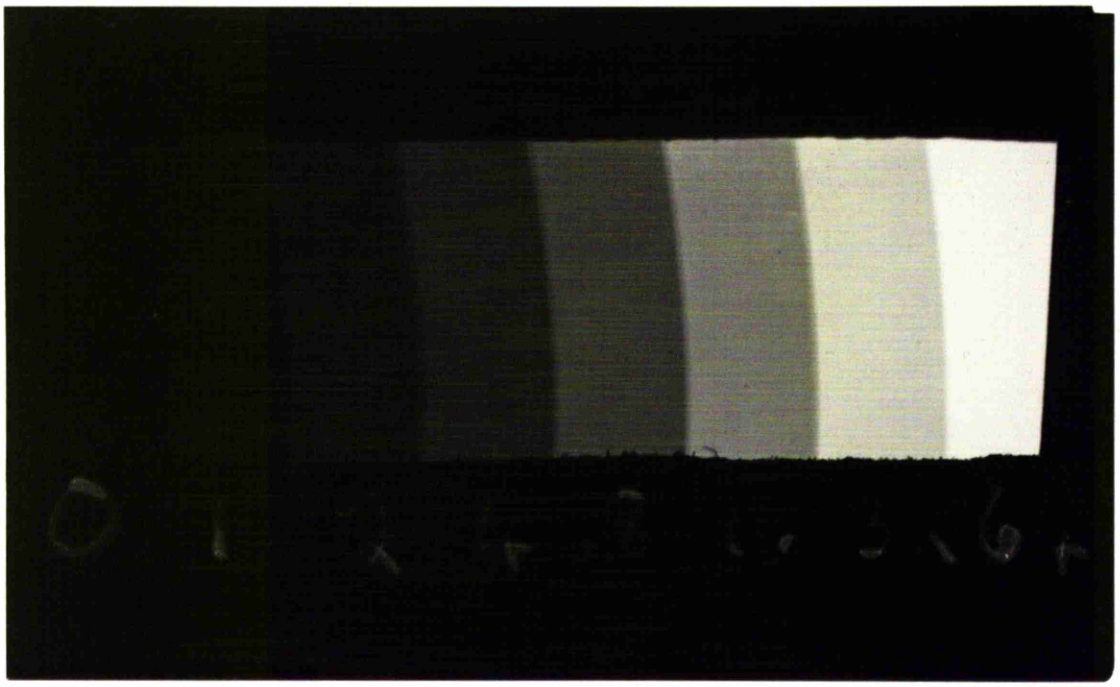

PLATE 9.

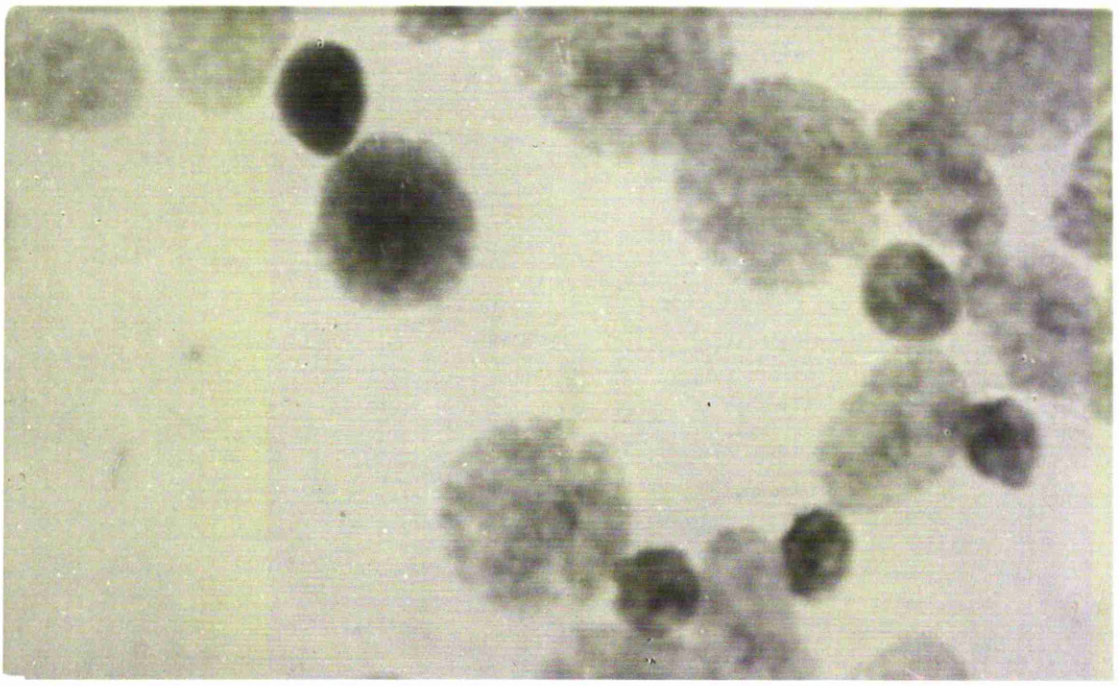

PLATE 10.

step-wedge is shown in Plate 9, the numbers indicating the relative exposure times received by the corresponding portions of the negative.

Plate 10 shows a typical field of nuclei from rat liver, after isolation in citric acid medium, while Plate 11 also shows rat liver nuclei, this time isolated in citric acid/sucrose medium. The greatly improved optical homogeneity is clearly shown.

Following on the criticisms of Naora (1951, 1952) that lenticular glare (the so-called Schwarzchild-Villiger phenomenon) might seriously affect the validity of microphotometric observations, a modified illumination system was devised to explore this possibility. The substage condenser was replaced by a high-power microscope objective, as described in Section 2.5a, which projected on to the object plane of the microscope an image of two closely adjacent small perforations in a metal plate located in front of the lamp iris diaphragm. The specimen slide was therefore illuminated at these two points only, and was positioned to include the whole of one nucleus in one illuminated spot, while the other covered a clear area of slide only. A number of exposures were made of single nuclei, under these conditions of minimum glare, a step-wedge being recorded on the film as before. Plate 12 shows such a photograph. (The apparently poor focus of the spots is due in

Plate 11.

Rat liver nuclei, Feulgen stained, after isolation in citric acid-sucrose medium. Note the greatly improved optical homogeneity of the nuclei, as compared with those isolated in citric acid (Plate 10).

Plate 12.

A rat liver nucleus, Feulgen stained, photographed by the "two-spot" illumination technique.

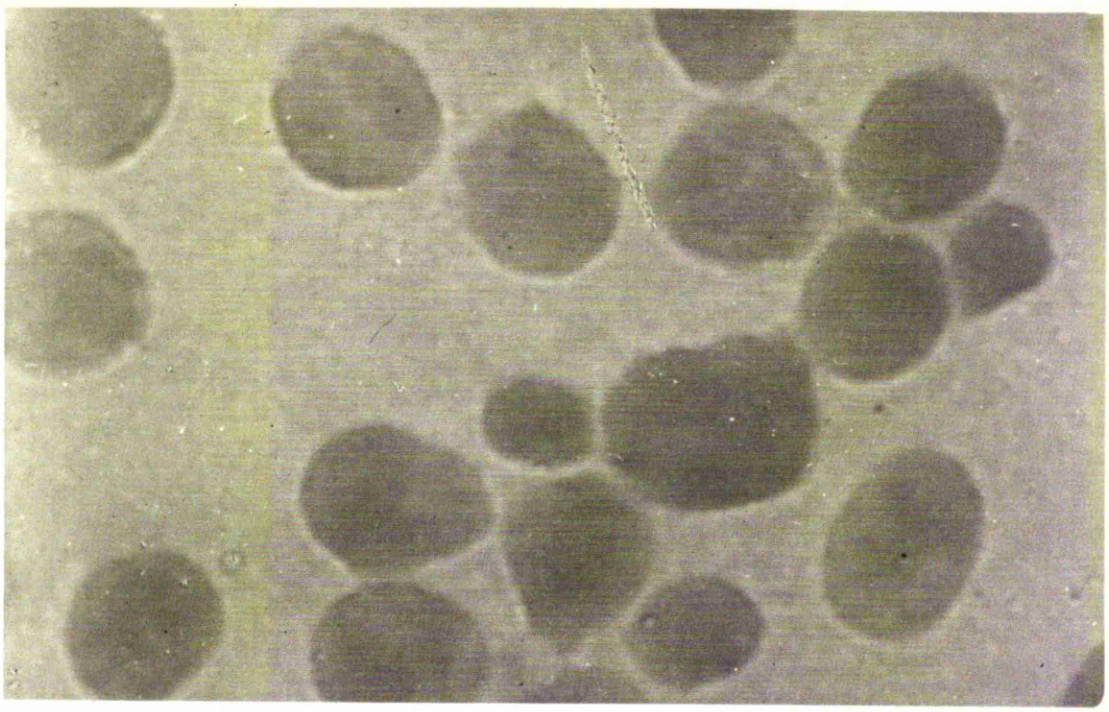

PLATE 11.

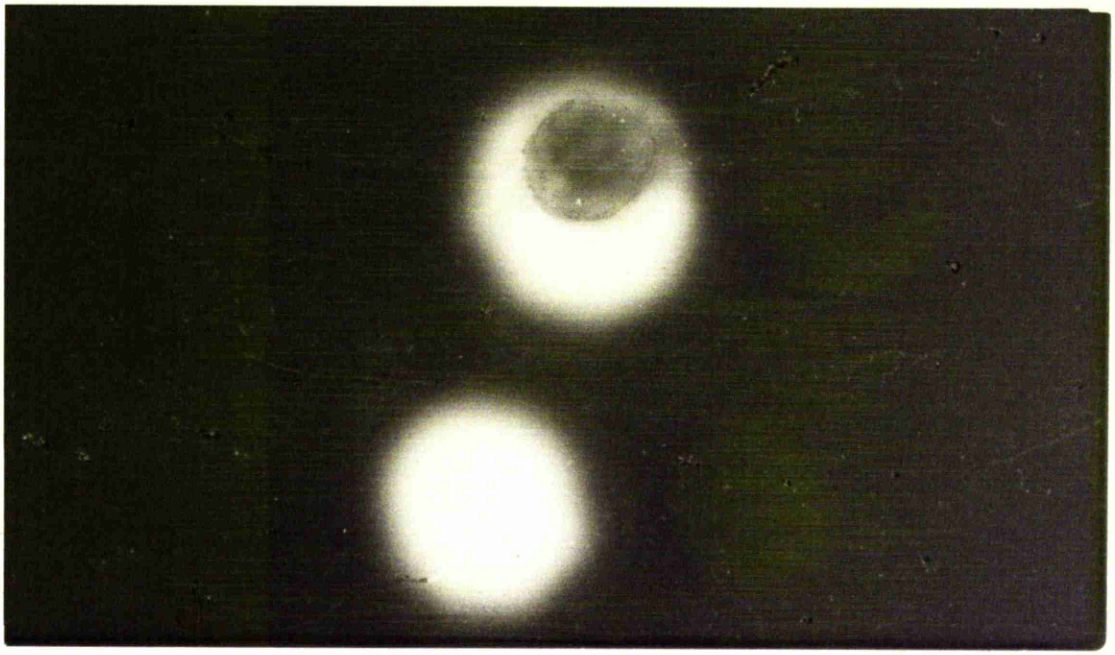

PLATE 12.

part to diffraction at the edges of the perforations themselves, the nuclear image being in normal focus.)

2.5d. Measurements on, and Calculations from, the Photographic Negatives.

The aim of the photographic method is to compare the intensity of light passing through a nucleus with that passing through an adjacent clear area of the slide, the ratio of these intensities being the transmission ("t") of the nucleus. The Extinction ("E") of a nucleus is given by $E = \log_{10} 1/t$, and is proportional to the total number of light-absorbing molecules, through which the light has passed. For a spherical or ellipsoidal homogeneous object, the total amount of light-absorbing material in the object is proportional to the product of the central extinction (E) of the object and its area (A), i.e.,

Amount of absorbing material $\propto$ E x A (Caspersson, 1940)

It is not necessary to know the thickness of the objects, provided they are spheres or regular ellipsoids, in order to compare the amounts of absorbing material which they contain, but the product of extinction and area gives a measure of total absorbing material in purely arbitrary units, which are therefore of significance only in comparing the amounts of material in two or more objects under the same conditions of measurement.

The majority of isolated nuclei are, for practical purposes, close to the required spherical shape, particularly those of liver, or can be regarded, at worst, as reasonably regular ellipsoids. It was considered permissible, in the present investigation, to assume that measurements based on the above relationships would not be seriously in error from this source. (An exception must be made in the case of rat sperm heads, which have a curved and flattened shape which is far from ideal for photometry. However, in the absence of alternative methods for measurement, it was decided to apply the same methods to sperm heads also, though with reservations as to their validity.)

It is therefore necessary, in order to measure the amount of light absorbing material in a nucleus, to measure the intensity of light passing through the centre of the nucleus, as compared with that reaching it (or reaching an equivalent clear area of the slide, if the field is evenly lit). It is also necessary to measure the area of the nucleus, in arbitrary units if comparative values only are required.

The procedure adopted for measuring the central extinctions of the nuclei was as follows:-

Measurements of light transmitted through the relevant portions of the negatives were made using a Hilger

photoelectric densitometer designed for spectrograph plate measurement. Instrument readings (galvanometer deflections in arbitrary units) were taken for three small areas of equal size, as near to the centre of each nucleus as possible, for two equal areas of empty background, one on either side of each nucleus, and for each step of the calibrating step wedge.

The readings obtained from the steps of the wedge were used to draw a calibration curve for that particular strip of film, in terms of units of film exposure against densitometer readings. From the mean of the three densitometer readings for each nucleus, a value for units of light transmitted by the central portion of the nucleus was read off from the curve, and similarly from the mean of the two background densitometer readings, a value for the incident light was obtained. From these two values, both in the same arbitrary units, the extinction of each nucleus was calculated.

For measurements of area of each nucleus, the negatives were enlarged to 30-40 diameters by a film-strip projector, and the outline of each nucleus was drawn on a sheet of paper by hand. The area of this projected outline drawing was then measured by a planimeter, and recorded in arbitrary units. Care was taken to ensure that all nuclei in a single film strip were drawn at the same degree of en-

largement, so that their areas were strictly comparable, but the arbitrary units of area were not necessarily comparable between experiments.

Then the product of the central extinction and the area of the projected enlarged nucleus was taken as the amount, in arbitrary units, of absorbing material contained in that nucleus. The measurement procedure was identical for negatives of Feulgen- and methyl green-stained nuclei, and for negatives obtained in ultraviolet light.

PART III.

THE RELATIVE DEOXYRIBONUCLEIC ACID CONTENT OF INDIVIDUAL CELL NUCLEI.

The Relative Deoxyribonucleic Acid Content of Individual Cell Nuclei.

3.1. Results by Chemical Estimation.

3.1a. The DNA content of isolated rat liver and kidney nuclei.

Table 1 presents the results of an experiment in which the numbers of nuclei present in aliquots of a sample of rat liver nuclei, isolated in citric acid-sucrose medium, were counted by haemocytometer, and the amount of DNA was found by estimation of the DNA phosphorus following chemical separation of the DNA fraction by the method of Schmidt and Thannhauser (1945). Results for kidney nuclei from the same animal are also presented. The mean values (0.85 pg DNAP per nucleus for kidney, and 1.20 pg DNAP per nucleus for liver) are somewhat higher than the average values found by Thomson, Heagy, Hutchison and Davidson (1953), although the ratio $\frac{\text{Mean DNAP per liver nucleus}}{\text{Mean DNAP per kidney nucleus}}$ (1.41 in the present experiment) corresponds closely with the average ratio calculated from their much more extensive data. Had the mean amounts found by Thomson *et al.* been substantially greater than those found in this experiment, it might have led to the suspicion that the methods employed by the author for nuclear isolation were permitting an appreciable loss of DNA from the nuclei. Since, however, the discrepancy is in the other direction, it appears more

Table 1.

The mean deoxyribonucleic acid phosphorus (DNAP) content of rat liver and kidney nuclei.

Tissue of origin	Number of nuclei in batch $\div 10^6$		DNAP in batch (µg.)	DNAP per nucleus (pg.)*	Ratio DNAP per liver nucleus / DNAP per kidney nucleus
	Separate counts	Mean			
Liver	841 839 810	830	997	1.20	1.41
Kidney	417 404 410	410	347	0.85	

* 1 pg. (picogram) = 10^{-6} µg.

Fractionation of nuclei by the method of Schmidt and Thannhauser (1945).

Phosphorus estimations by the method of Allen (1940).

probable that the nuclei in the author's preparations were contaminated to a small extent with nuclear fragments containing DNA. This contamination, if present, should not interfere with the main photometric estimations to be reported later in the present section. Duplicate experiments in which nuclei were isolated in the sucrose-citric acid medium (described in Section 2.3a) gave values for the mean DNAP content slightly higher in every case than those published by Thomson et al., while an experiment, in which the nuclei were isolated in citric acid medium only, gave results for kidney and liver which are more nearly in accord with published values. It has already been indicated, however, that nuclei, isolated in citric acid only, are optically inhomogeneous and unsuited to photometric estimations.

3.1b. The influence of ribonuclease treatment on the composition of isolated rat cell nuclei.

It is by now well known that the cell nucleus contains ribonucleic acid in addition to the predominant deoxyribonucleic acid (Brachet, 1941; Davidson and Waymouth, 1944). Since it was intended in the present experiments to estimate the DNA content of isolated nuclei by ultraviolet absorption methods, and since RNA absorbs ultraviolet light similarly to DNA, it was considered desirable to carry out a preliminary study of whether such RNA could be effectively

removed from isolated nuclei prior to their U-V study. Table 2 shows the amounts of DNA phosphorus and RNA phosphorus present in an aliquot of a preparation of isolated rat liver nuclei, as compared with the amounts present in aliquots which had been treated with solutions of crystalline ribonuclease, under the conditions of incubation detailed in Section 2.4d, for periods of one and four hours. It will be seen that treatment with ribonuclease substantially diminishes, but does not remove completely, the material contained in the Schmidt-Thannhauser fraction usually regarded as consisting of RNA phosphorus. This is in accord with the observations of Davidson and Smellie (1952) that the so-called ribonucleotide fraction obtained in tissue analyses by the method of Schmidt and Thannhauser includes substantial amounts of phosphorus-containing compounds other than those derived from RNA, and therefore presumably not removable by treatment with ribonuclease. From this experiment, however, one may conclude that ribonuclease treatment reduced the apparent RNAP/DNAP ratio, in this batch of nuclei, from 1:7 to 1:12. If one may assume that the compounds which remain as "apparent RNA phosphorus" show relatively less intense ultraviolet-absorbing properties around 2600 A than do RNA and DNA, then ribonuclease treatment should, in fact, reduce the U-V absorption interference very substantially. This conclusion is supported by the

Table 2.

The influence of ribonuclease on the composition of isolated rat liver nuclei.

Treatment	Amount of DNA phosphorus in batch (µg.)	Amount of RNA phosphorus in batch (µg.)	$\frac{\text{DNAP}}{\text{RNAP}}$
Control (nuclei incubated in buffer)	323	44.5	7.3:1
Nuclei incubated in buffer plus ribonuclease 0.1 mg./ml., for 1 hr.	309	25.5	12.1:1
Nuclei incubated in buffer plus ribonuclease 0.1 mg./ml., for 4 hrs.	312	24.0	13:1

Fractionation of nuclei by the method of Schmidt and Thannhauser (1945).

Phosphorus estimations by the method of Allen (1940)

microspectrophotometric results reported in the next section. It is also apparent from Table 2 that prolonged incubation with ribonuclease has little advantage over one-hour incubation, under optimum conditions in both cases.

3.2. Results by Microspectrophotometry of Rat Liver and Kidney Nuclei.

3.2a. The relative deoxyribonucleic acid content of rat liver and rat kidney nuclei, as measured by their absorption of U-V light.

Figure 6 presents in the form of frequency histograms, the amounts of ultraviolet-absorbing material found in individual liver and kidney nuclei isolated from the same adult rat. The upper part of the figure applies to nuclei which were measured without enzymic treatment, and therefore presumably containing RNA in addition to DNA, while the lower part of the figure refers to nuclei from the same batches, which had been submitted to ribonuclease digestion prior to ultraviolet photography and measurement. The measurements were made on photographic negatives exposed at 2536 A, which is reasonably close to the absorption maximum for the nucleic acids (2600 A), and the upper portion of the figure represents the amounts, in single nuclei, of all substances which absorb U-V light at that wavelength. In practice, however, nuclei isolated in an aqueous medium, fixed in acetic-alcohol, and washed, are unlikely to contain any ultraviolet-absorbing material other than the nucleo-

Figure 6.

Frequency histograms of the relative amounts of ultraviolet-absorbing material contained in individual rat kidney and rat liver nuclei, as estimated by photometric measurements of their absorption of ultraviolet light (λ = 2536 A)

A) without enzyme treatment

B) with ribonuclease treatment prior to measurement.

Class interval = 40 units.

(N.B. The arbitrary units used in this figure are not identical with those used in the other figures.)

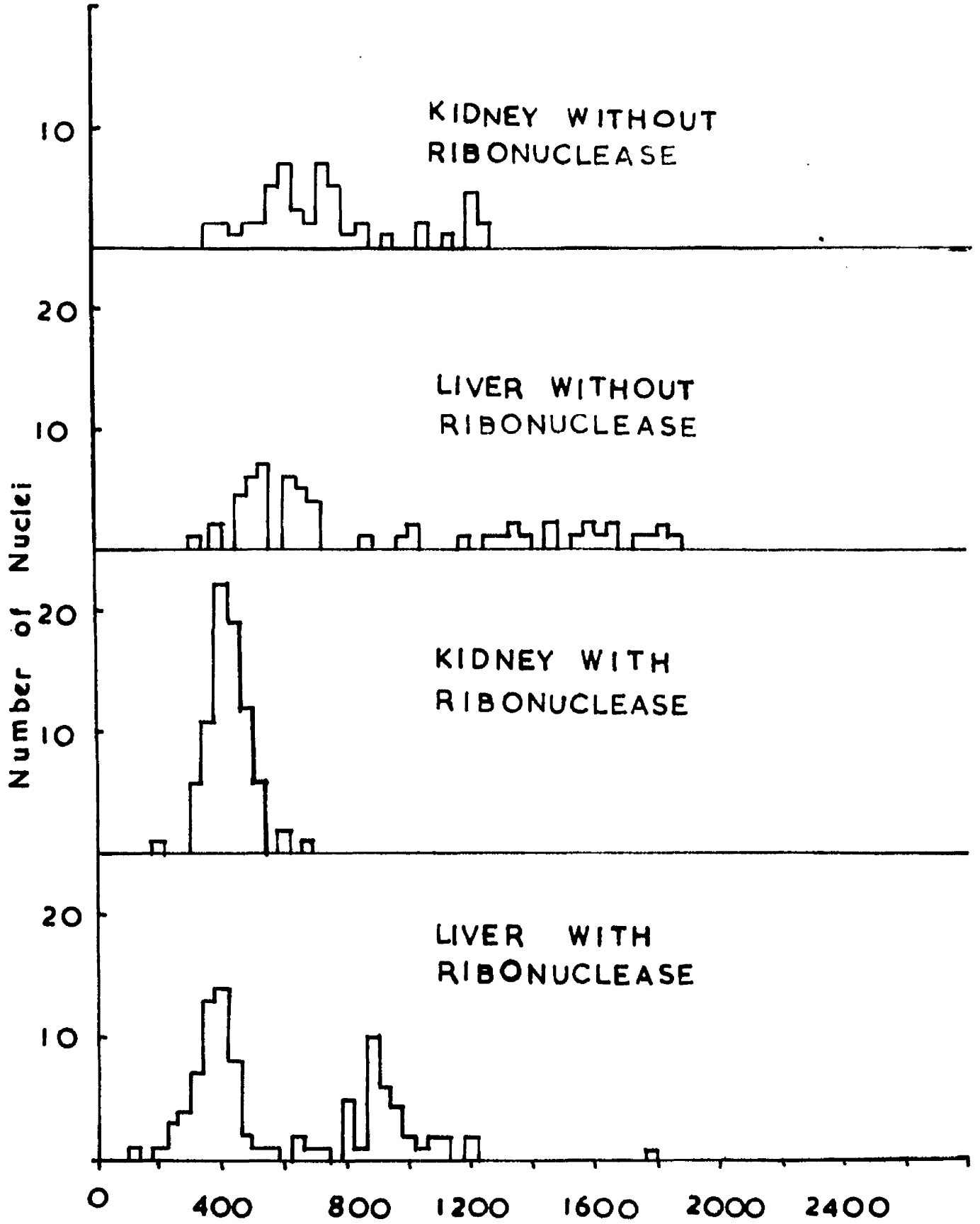
KIDNEY WITHOUT RIBONUCLEASE
LIVER WITHOUT RIBONUCLEASE
KIDNEY WITH RIBONUCLEASE
LIVER WITH RIBONUCLEASE
Number of Nuclei
10
20
10
20
10
20
10
0
400
800
1200
1600
2000
2400
Amount of ultraviolet-absorbing material per nucleus. (arbitrary units)

Figure 6.

proteins. After ribonuclease digestion, only deoxyribonucleoprotein is likely to be present in significant amounts, and therefore the lower portion of Figure 6 represents the amounts of DNA (plus protein) in each nucleus measured.

The upper portion of Figure 6 indicates that the 55 kidney nuclei range in their content of U-V absorbing material from 350 to 1200 units, with a mean value of 725 units, while 63 liver nuclei, having a mean of 889 units, range in individual values from 300 to 1800 units.

(It is important to note that the units of measurement are purely arbitrary, and apply within one experiment only, although the relative values for, say, liver and kidney nuclei in the same strip of negatives should be comparable. The same considerations apply to the units of Feulgen dye or methyl green dye recorded in subsequent experiments - the units inevitably differ from one set of measurements to another, and are only comparable within a single experiment. The use of kidney nuclei as a standard of comparison on each slide, however, should allow comparisons between the experiments even although the units of measurement differ.)

In the lower half of Figure 6 are recorded comparable measurements on nuclei from the same batches, but treated with ribonuclease. 81 kidney nuclei, whose mean content of U-V absorbing material is 382 units, range in values

from 300 to 500 units, with only four nuclei falling outside these values.

The 105 liver nuclei measured, and having a mean value of 611 units, range from 120 units to nearly 1800 units. From the frequency histogram, however, it is clear that while the kidney nuclei almost all come within a single symmetrical peak, the liver nuclei can be regarded as forming two main groups, with the exception of a single nucleus having a much higher content of absorbing material. If 600 units is arbitrarily adopted as the dividing line between the two main groups, then 58 nuclei form the first group, having a mean content of 376 units, while 46 nuclei form the second group, which has a mean value of 881 units. The solitary nucleus with a much higher value (1760 units) might be regarded as representing a third group still.

The influence of ribonuclease upon the total ultraviolet absorbency of the nuclei is striking. Not only does the enzymic treatment reduce the total amount of U-V-absorbing material in the nuclei, but this removal reveals a pattern in the frequency with which particular amounts of absorbing material are found within single nuclei. If it is accepted that the U-V absorption after removal of RNA is primarily a measure of the amount of DNA present, then one must conclude that certain quantities of DNA are found more frequently than appears likely on grounds of random

variation only. Further, it is implied, by this finding, that the material removed during digestion with ribonuclease is of much more variable distribution amongst the nuclei than is the DNA itself. Had the amounts of this removable material in individual nuclei been in any way related to the amounts of DNA present in the same nuclei, then the wide and largely random scatter of values shown in the upper half of Figure 6 would not be likely to be found.

3.2b. The relative deoxyribonucleic acid content of rat liver and rat kidney nuclei, as measured by microphotometry following Feulgen staining.

Similar frequency histograms for rat kidney nuclei and rat liver nuclei, in terms of their content of Feulgen dye, are presented in Figure 7. These values were obtained from nuclei from the same batches as were employed in the ultraviolet experiments whose results are recorded in Figure 6, and therefore comparisons between the two sets of results should be valid. It must again be emphasised, however, that the units of Feulgen dye content, though comparable as between the liver and kidney nuclei in this experiment, are quite arbitrary and not directly comparable with the units of U-V absorbing material reported in Section 3.2a, nor are they comparable directly with the Feulgen dye units in other experiments.

Figure 7.

Frequency histograms of the relative deoxyribonucleic acid (DNA) contents of individual rat kidney and rat liver nuclei (first batch), as estimated by photometric measurements following Feulgen staining.

Class interval = 40 units.

(N.B. The arbitrary units of the amounts of DNA per nucleus are applicable to this figure only, and are not comparable directly with those in the other figures.)

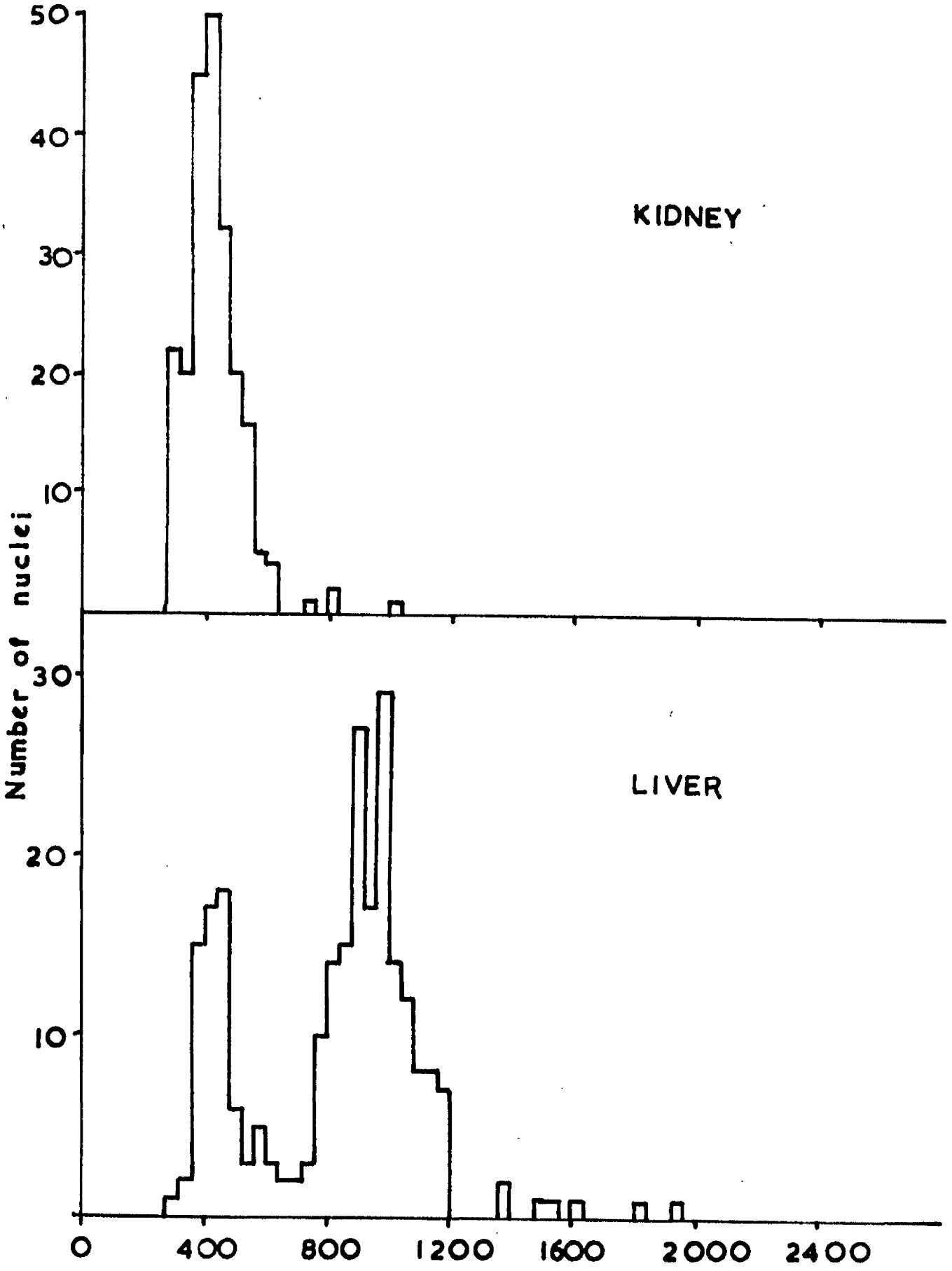
KIDNEY
LIVER
Number of nuclei
50
40
30
20
10
30
20
10
0
400
800
1200
1600
2000
2400
DNA content per nucleus (arbitrary units)

Figure 7

In Figure 7, values for 224 kidney nuclei are recorded, and fall about a mean value of 443 units of DNA content. Practically all the kidney nuclei are in the range 300-600 units, while a very few fall in the range 600-1000 units. For liver nuclei, however, the picture is very different; the 234 liver nuclei measured have a mean value of 830 units of DNA, but it is clear that the nuclei fall into distinct groups as regards their content of Feulgen dye. Thus 61 nuclei, in the range 650 units or less, fall fairly symmetrically around a mean of 443 units, while 165 nuclei, in the range 650-1300 units, have a mean value of 941 units. The remaining 8 nuclei, with values of 1300 units or more, give a mean of 1597 units.

We thus find, when several hundred nuclei are measured by the Feulgen technique, that almost all the kidney nuclei form a single group as regards their content of DNA, while the population of liver nuclei can be readily subdivided into a first group whose mean DNA content corresponds closely to the mean value for the kidney nuclei; a second, and larger, group, having a mean value approximately twice that of the lower group; and a third, much smaller group, with higher values still. This latter group is too small for its mean value to be of much significance, but it approximates to twice that of the main group of liver nuclei, or four times that of the kidney nuclei.

In order to verify that this pattern of results did not represent a departure from the normal state of affairs, a second series of measurements was carried out on entirely separate batches of kidney and liver nuclei obtained from another animal. The results of this second experiment are recorded in Figure 8. Without detailing the numbers of nuclei and the mean values for the different peaks in this figure, one can see that the kidney nuclei again form a compact and symmetrical group, with in this case only two nuclei having values outside the main group. The liver nuclei again form two main classes, one having approximately the same mean value for DNA content as that found for kidney nuclei, and the second having a mean value approximately twice that of the lower group. Again, a few liver nuclei show much higher values still, and may be arbitrarily taken together to form a third class. The most noteworthy difference between the results presented in Figures 7 and 8 is the difference in relative sizes of the two main classes of liver nuclei in the two experiments. Thus in Figure 7, the group lowest in DNA content (henceforth called "Class I") is considerably smaller than the second group ("Class II"), whereas, in Figure 8, Class I nuclei are considerably more numerous than those in Class II. The small number of nuclei giving higher values ("Class III") is too small for comparison of numbers. One cannot, however, conclude that

Figure 8.

Frequency histograms of the relative deoxyribonucleic acid (DNA) contents of individual rat liver and kidney nuclei (second batch), as estimated by photometric measurements after Feulgen staining.

Class interval = 40 units

(N.B. The arbitrary units of DNA content used in this figure are not identical with those used in the other figures. The nuclei were from a different animal from those represented in Figure 7.)

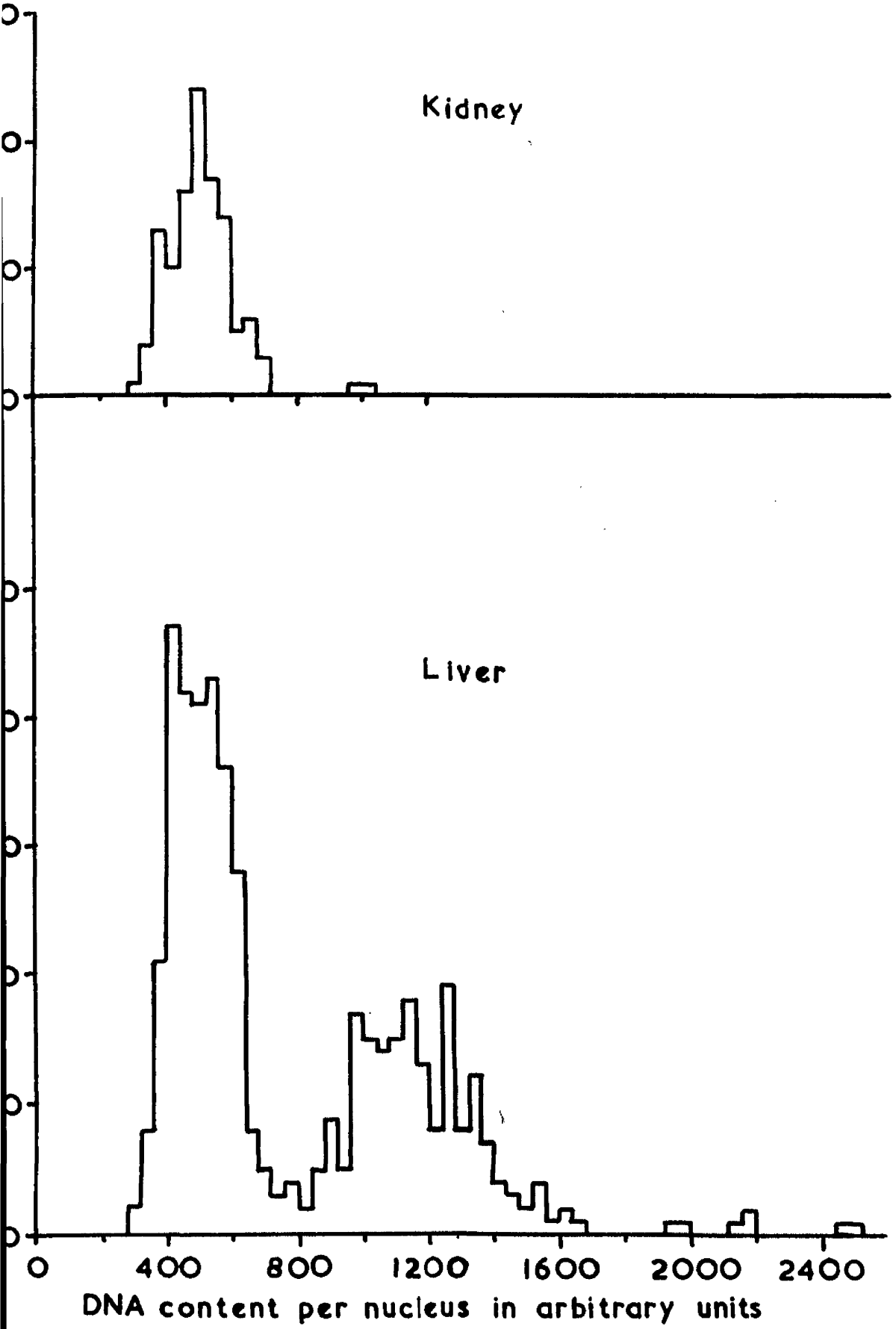
Kidney
Liver
0
400
800
1200
1600
2000
2400
DNA content per nucleus in arbitrary units

Figure 8.

the two animals necessarily differed greatly in the relative numbers of the two main classes of nuclei present in their livers, since one cannot possibly regard the very small number of nuclei actually measured as constituting a valid sample of the nuclei present in the intact animal. Selection may well have arisen at several stages, not least at the stage when nuclei, of shapes suitable for measurement on the photographic negatives, are being chosen and numbered.

When the values for the liver nuclei were calculated, it became clear that there was at least a partial correlation between the size of a nucleus and its measured content of DNA. Figure 9 shows this relationship for the liver nuclei whose DNA contents are recorded in Figure 7, the DNA content being plotted against the area of the nucleus in arbitrary planimeter units. The small liver nuclei, known to be derived mainly from the bile ducts, blood vessels, Kupffer cells and other non-parenchymal sources, without exception fall into Class I, corresponding in DNA content to the kidney nuclei, while the great majority of the larger nuclei, which in histological sections can be readily identified as the nuclei of liver parenchymal cells, fall into Class II. The few nuclei having a very high DNA content are also large nuclei, though not necessarily large in proportion to their content of DNA.

Figure 9.

The relationship between the size of rat liver nuclei and their content of deoxyribonucleic acid, as estimated by Feulgen staining.

(Measurements on the liver nuclei recorded in Figure 7.)

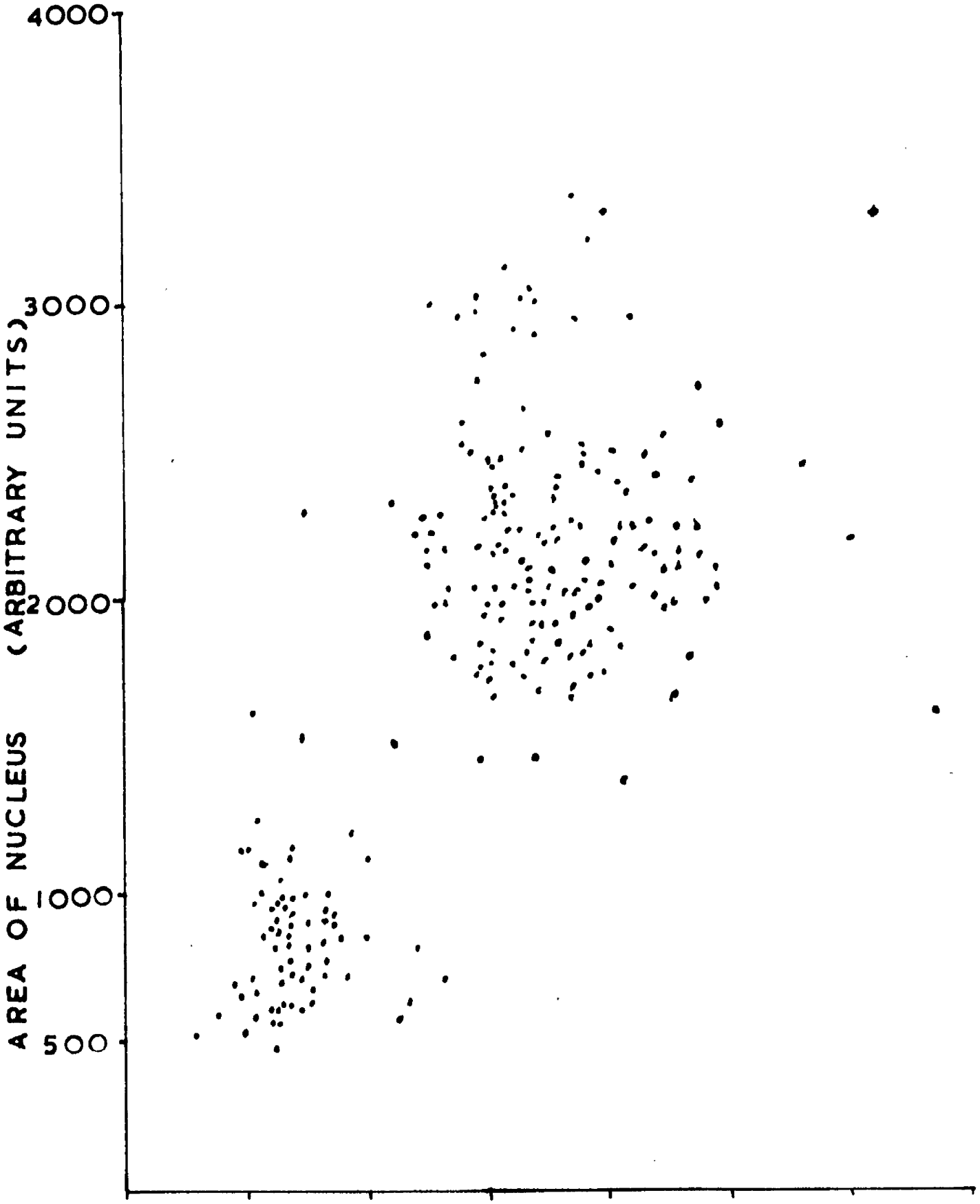
4000
3000
2000
1000
500
AREA OF NUCLEUS (ARBITRARY UNITS)
200
400
600
800
1000
1200
1400
DNA content of nucleus (arbitrary units)

Figure 9.

3.2c. The relative deoxyribonucleic acid content of rat liver and rat kidney nuclei, as measured by microphotometry following methyl green staining.

Frequency histograms showing the DNA content of rat kidney and rat liver nuclei, as measured by the methyl green staining method, are shown in Figure 10. The nuclei used in this experiment were again from the same animal as those whose DNA contents are presented in Figures 6 and 7. The actual units of measurement, however, apply to this experiment only.

Again the kidney nuclei form a single, approximately symmetrical peak, the 106 nuclei measured having a mean value of 278 units. The 159 liver nuclei, with an over-all mean value of 436 units, can be subdivided into 80 nuclei, with values from 0-400 units about a mean of 288; 73 nuclei, containing from 400-800 units, mean value 546, and 6 nuclei, having more than 800 units, with a mean of 1091.

Although the division between the classes is less distinct in this case than that found in the Feulgen and U-V measurements, the general resemblance between the frequency histograms obtained in the three main types of microphotometric experiments is noticeable. Since all were performed on nuclei from the same batches, obtained from a single animal, it appears not unlikely that all three techniques are measuring the same substance. For ease in manipulation, however, subsequent microphotometric experiments were carried

Figure 10.

Frequency histograms of the relative deoxyribonucleic acid (DNA) contents of individual rat kidney and liver nuclei, as estimated by photometric measurements after methyl green staining.

Class interval = 40 units.

(N.B. The arbitrary units of DNA content used in this figure are not identical with those used in the other figures.)

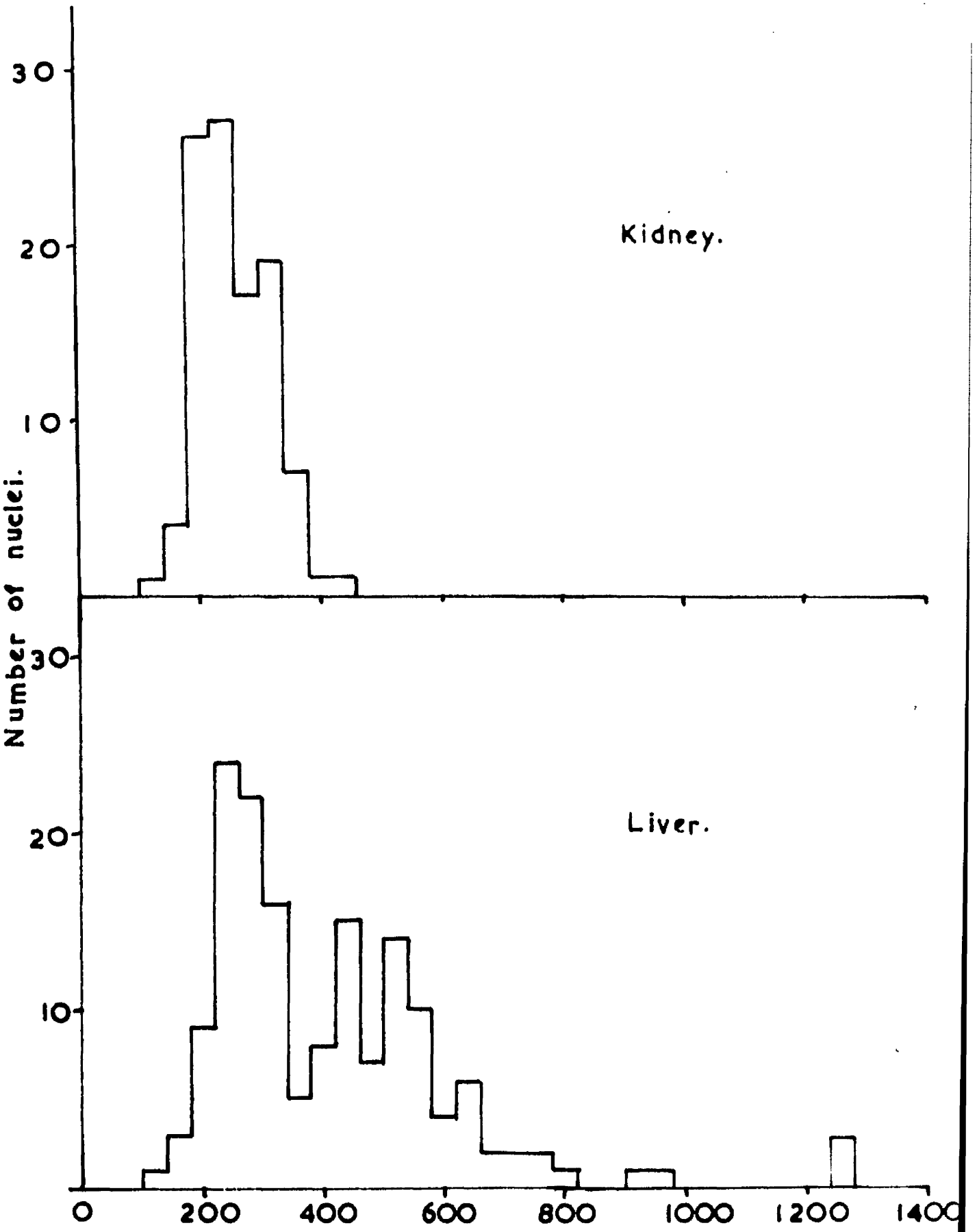
30
20
10
Number of nuclei.
Kidney.
30
20
10
Liver.
0
200
400
600
800
1000
1200
1400
DNA content per nucleus (arbitrary units)

Figure 10.

out on Feulgen-stained material only.

3.2d. The relative proportions of different cell types in rat liver.

It is clear from the experiments so far recorded that rat liver contains at least three classes of cell nuclei, as judged by the amounts of DNA which they contain, these amounts being in the approximate ratio 1 : 2 : 4. Histologists have also recorded that by chromosome counts it is possible to subdivide rat liver nuclei into diploid, tetraploid, octoploid and sextuploid, i.e., nuclei containing 2, 4, 8, and 16 times the number of chromosomes contained in the gametes. Some confusion has arisen, however, from failure to distinguish between liver nuclei, i.e., all types of nuclei found in normal liver, and hepatocyte nuclei, i.e., the nuclei of liver parenchymal cells. Biesele (1944) however, gives the relative proportions as approx. 54% diploid, 40% tetraploid and 5% octoploid. For purposes of comparison and calculation in the present experiments, differential counts were also made of the numbers of hepatocyte and non-hepatocyte nuclei seen in microtome sections of normal rat liver, from different animals. The results were as follows:-

	Animal 1	Animal 2
Hepatocyte nuclei	288 (58%)	366 (61%)
Other nuclei	212 (42%)	234 (39%)

i.e., as a near approximation, 60% of the nuclei in normal

rat liver are hepatocyte nuclei, while 40% are nuclei of the various supporting cells, ducts, sinusoids etc. If we make the assumption that the Class I nuclei recorded in Figures 6, 7, 8 and 10 are non-hepatocyte nuclei, while the Class II nuclei are derived from hepatocytes, then it is possible to calculate, from the photometric measurements, the overall mean value for DNA per kidney nucleus as compared with the overall mean value for DNA per liver nucleus, and to compare the ratio with that found for liver and kidney by direct chemical estimation. That the necessary assumption may not be fully justified is clear from comparison of Bieselo's figures for diploid and tetraploid nuclei, with the differential counts quoted above. If both were made on comparable livers, then a considerable proportion of the hepatocyte nuclei must certainly be diploid. If we take Bieselo's proportions for diploid, tetraploid and octoploid nuclei, and the mean values for Class I, II and III nuclei from the data of Figure 7, then the ratio

$$\frac{\text{Mean DNA per liver nucleus}}{\text{Mean DNA per kidney nucleus}}$$ is found to be 1.6:1.

If, on the other hand, we assume that all hepatocytes fall into Class II, while all non-hepatocytes fall into Class I, then the corresponding ratio, based on the differential counts quoted above, is 1.67:1. This compares with the ratio 1.41:1 found in Section 3.1a for the same batches of nuclei by direct chemical estimation, and suggests that

the batch of liver nuclei on which the chemical estimation was made may not itself have been a representative sample of all the types of nuclei present in the intact liver tissue. The larger Class II nuclei, for example, may have been selectively damaged during the initial homogenisation of the tissue, so that the final preparation contained an undue proportion of the smaller Class I nuclei, which, as we have seen, contain smaller amounts of DNA than do the larger nuclei.

3.2e. The influence of lenticular glare on microspectrophotometric measurements.

Naora (1951, 1952) has claimed that the majority of cytophotometric measurements are seriously in error on account of what is called the Schwarzschild-Villiger phenomenon. This effect is better known to microscopists in this country as lenticular glare, and consists of the re-introduction, into the visible field, of light which has been scattered at optical surfaces in the light path. The phenomenon is most likely to be detectable when large-aperture, multi-component, dry working objective lenses and substage condensers are used together, and above all when the effective aperture of the condenser exceeds that of the objective. The result of such glare, if present, will be to diminish the contrast of the microscope image, or, in photometric terms, to cause the optical density of the specimen to be

underestimated. Figure 11 presents the results of an experiment designed to test whether this phenomenon was in fact causing serious difficulty in the present series of measurements. 70 rat liver nuclei in a Feulgen-stained preparation were measured with respect to their DNA content under the standard optical conditions described in Section 2.5a., i.e., with the whole field illuminated in the normal manner. The upper half of Figure 11 shows that this group of liver nuclei formed the expected two classes, the mean value for the Class II peak being, as before, approximately twice that for the Class I peak.

The same 70 nuclei were then re-measured under "two-spot" illumination conditions, achieved as described in Section 2.5a. Here the illumination of the object plane is restricted to two areas of about 10 μ in diameter, one being positioned to include the nucleus being measured, and the other an appropriate area of clear background: such illumination should obviously greatly diminish the lenticular glare, if any is present, and might therefore be expected to increase the measured extinctions of the nuclei, with a corresponding shift of the peaks to the right. It is clear, however, from the lower half of Figure 11 that the results given by the two methods of illumination are very similar; in particular, there has not been any systematic increase in the extinctions found for this group of

Figure 11.

Frequency histograms of the relative deoxyribonucleic acid (DNA) contents of the same group of rat liver nuclei, as estimated by photometric measurements after Feulgen staining,

A) with "whole-field" illumination of the specimen;

B) with restricted "two-spot" illumination.

Class interval = 100 units.

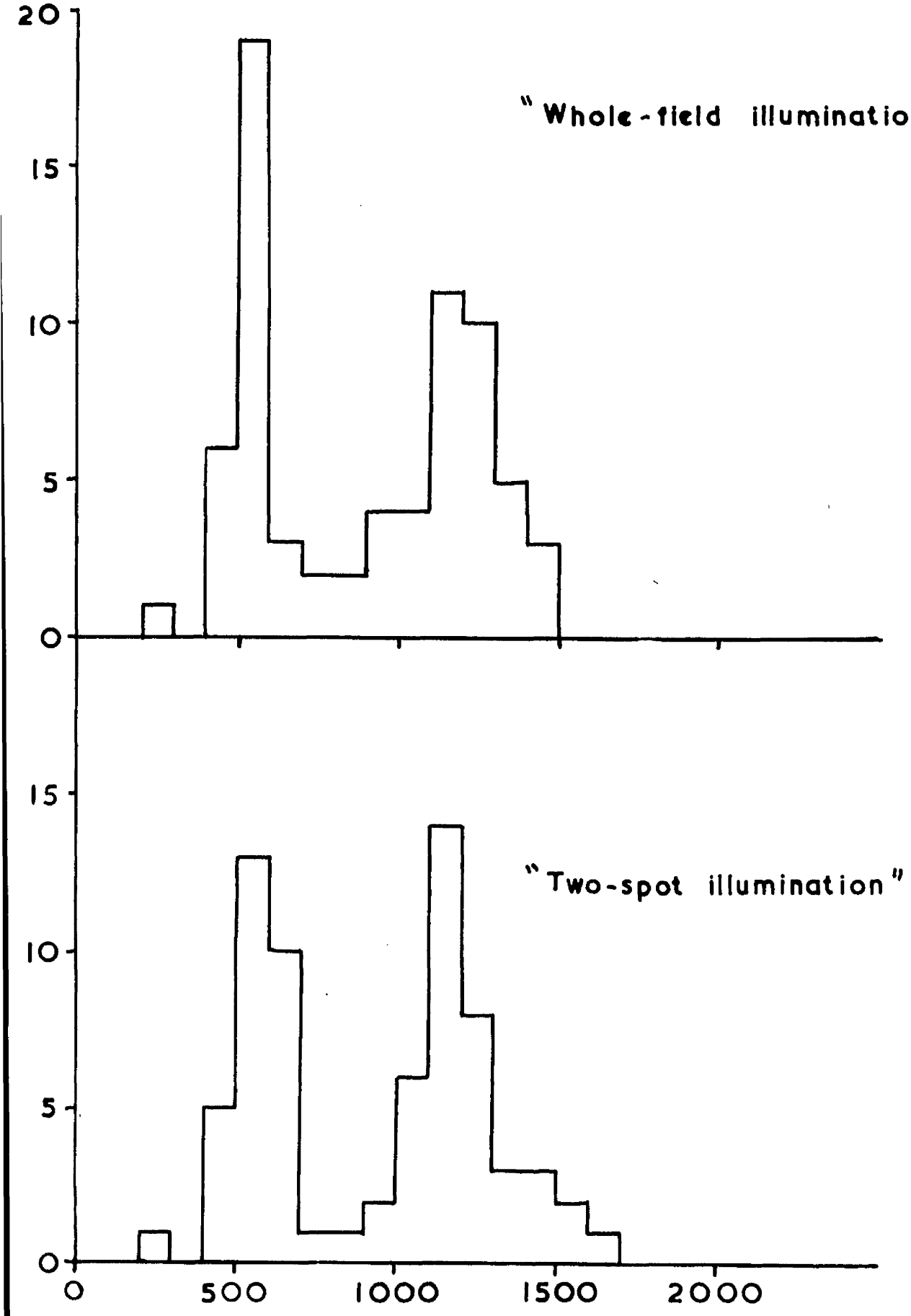
20
15
10
5
0
"Whole-field illumination"
15
10
5
0
"Two-spot illumination"
0
500
1000
1500
2000
DNA content per nucleus in arbitrary units

Figure 11.

nuclei. Study of the individual extinctions shows that in some cases the second measurement is higher than the first, in others the reverse is true, the discrepancies being related to the general errors of such measurements and not to a systematic error of the sort described by Naora. There is nothing in the results of this experiment to suggest that normal whole-field illumination is introducing serious error from lenticular glare, a conclusion also supported by Ornstein and Pollister (1952).

3.3. Results by Microspectrophotometry of Nuclei from Rat Tissues other than Normal Liver and Kidney.

3.3a. The DNA content of nuclei in rat intestine, pancreas and spermatozoa.

Before any conclusions can be drawn regarding the significance, if any, of the occurrence of more than one class of nucleus in rat liver, it is clearly desirable to carry out a series of measurements on other rat tissues with a view to ascertaining whether liver is unique in this respect. The chemical measurements of Thomson, Heagy, Hutchison and Davidson (1953) on a variety of rat tissues suggest that nuclei from kidney, and most other tissues except liver, have approximately the same average DNA content per nucleus. Cytophotometric measurements were therefore carried out on nuclei isolated from rat intestine and rat pancreas, in an attempt to find whether the constancy of average DNA content

found by bulk chemical methods would also be shown by photometry of single nuclei. Figure 12 presents a series of comparable measurements on Feulgen-stained nuclei from small intestine and pancreas, with a comparison standard of kidney nuclei from the same animal. The units of DNA content are comparable between the three tissues, but again are not comparable directly with the units in other similar experiments. 75 kidney nuclei, with a mean DNA content of 419 units, form a single symmetrical peak. 88 small intestine nuclei, mean DNA content 411 units, are again all contained in a single peak, presumably corresponding to the Class I peak of liver, with the exception of one nucleus, whose value of 780 units appears to belong to Class II. 213 pancreas nuclei, with a mean value of 515 units, can readily be seen to fall into two distinct groups, the mean value for the first peak being close to that for the kidney and small intestine nuclei. If 600 units, the upper limit for the kidney nuclei in this experiment, is taken as the upper boundary level for Class I nuclei, then the pancreas nuclei can be subdivided as follows:-

174 Class I nuclei, 82% of the sample, mean value 439 units,

and

39 Class II nuclei, 18% of the sample, mean value 858 units.

Thus we see that while the nuclei of small intestine, like those of kidney, form a single Class I group, those

Figure 12.

Frequency histograms of the relative deoxyribonucleic acid (DNA) contents of individual nuclei of rat kidney, small intestine, and pancreas, as estimated by photometric measurements after Feulgen staining.

Class interval = 20 units.

(N.B. The arbitrary units of DNA content are applicable to this figure only.)

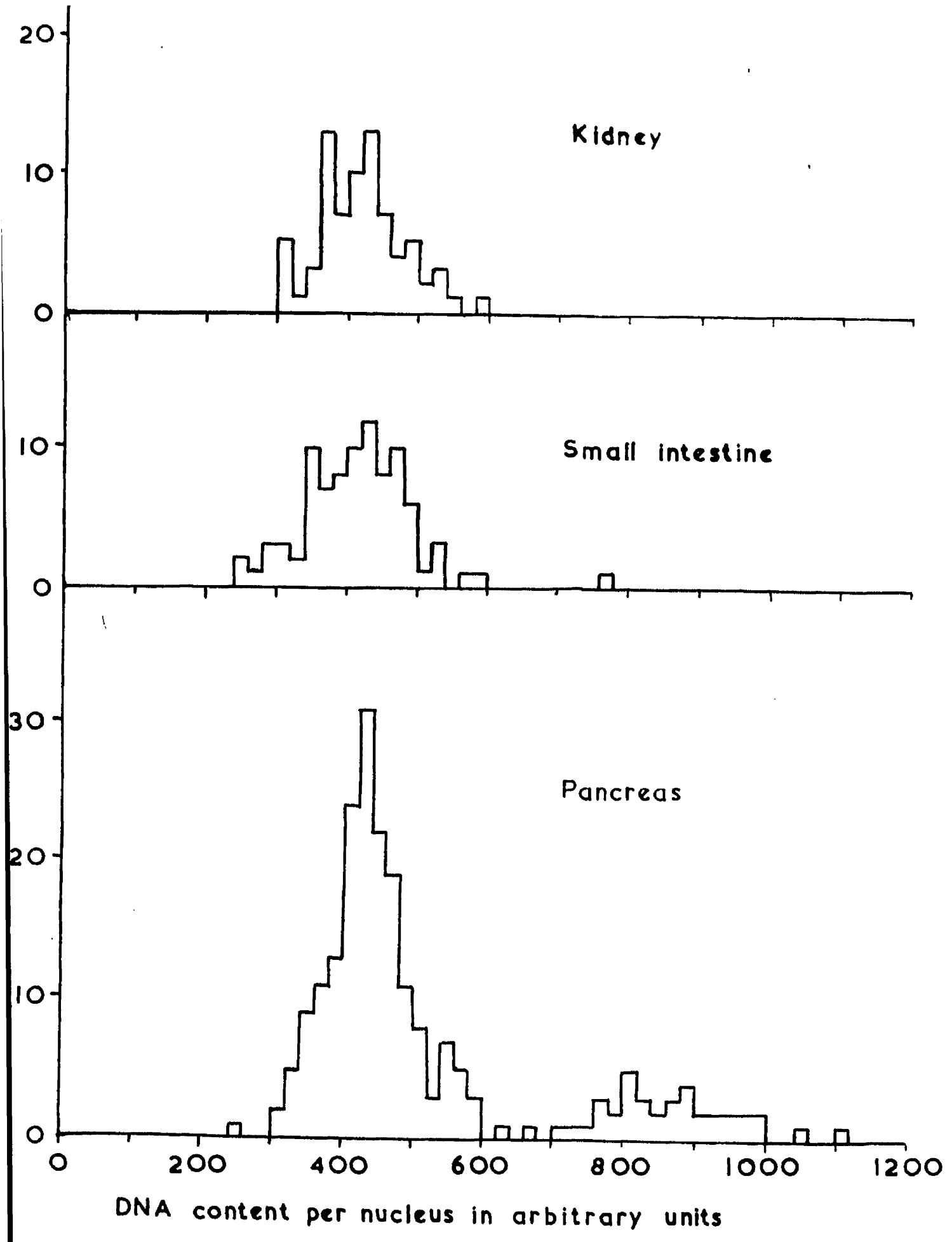
20
10
0
Kidney
10
0
Small intestine
30
20
10
0
Pancreas
0
200
400
600
800
1000
1200
DNA content per nucleus in arbitrary units

Figure 12.

from pancreas resemble liver nuclei in forming Class I and Class II groups, the latter having approximately twice the DNA content of the Class I nuclei. In the case of pancreas, however, the Class II nuclei form a much smaller proportion of the total nuclei than is the case in liver. In the present experiment, there is no indication of a Class III group in pancreas, although the sample measured is hardly large enough to justify the conclusion that no such group exists. The occurrence of a single Class II nucleus, for example, in the small intestine sample measured, might readily have been missed, and led to the assumption that all intestinal nuclei belong to Class I.

The question of the relationship between DNA content of nuclei and their complement of genetic material is of very considerable interest, and therefore it seemed desirable to attempt similar measurements on the gametes, which, as is well known, contain only one set of chromosomes as compared with the minimum of two sets occurring in the typical somatic nucleus. The mammalian ovum is not readily available for study in quantity, nor is it altogether suitable for photometric study, the large nucleus, staining feebly by the Feulgen technique, being almost outside the working limits of microspectrophotometry. Spermatozoa, however, are readily available in amounts sufficient for photometric study by the methods used in the present experiments, the

difficulty being that the rat sperm head is a slender densely staining sickle-shaped object quite unlike the spherical nucleus which is desirable for photometry. However, the importance of knowledge of the DNA content of individual spermatozoa is such that it was considered justifiable to make a series of measurements on sperm heads, even although the calculated values resulting from such measurements might well be less reliable than those from the other tissues studied. Figure 13 presents the results of such measurements on rat spermatozoa, compared with a series of measurements on kidney nuclei isolated from the same animal. 72 rat sperm heads gave a mean value of 195 units of DNA, while 113 kidney nuclei had a mean value of 337 units. It is noteworthy that the scatter of values amongst the sperm heads is lower than in any group so far measured. The ratio of the mean DNA content of the sperm heads to the mean DNA content of the Class I nuclei found in kidney is, in this experiment, 0.59/1, an appreciable divergence from the 0.5/1 ratio which might have been predicted.

3.3b. <u>The DNA content of nuclei in regenerating liver and in tumour-bearing liver.</u>

In a study concerned with the relationship between the functions of cell nuclei and their content of DNA, it would be desirable to study the relationship between the

Figure 13.

Frequency histograms of the relative deoxyribonucleic acid (DNA) contents of individual rat kidney nuclei and rat spermatozoa, as estimated by photometric measurements after Feulgen staining.

Class interval = 20 units.

(The arbitrary units of DNA content are applicable to this figure only.)

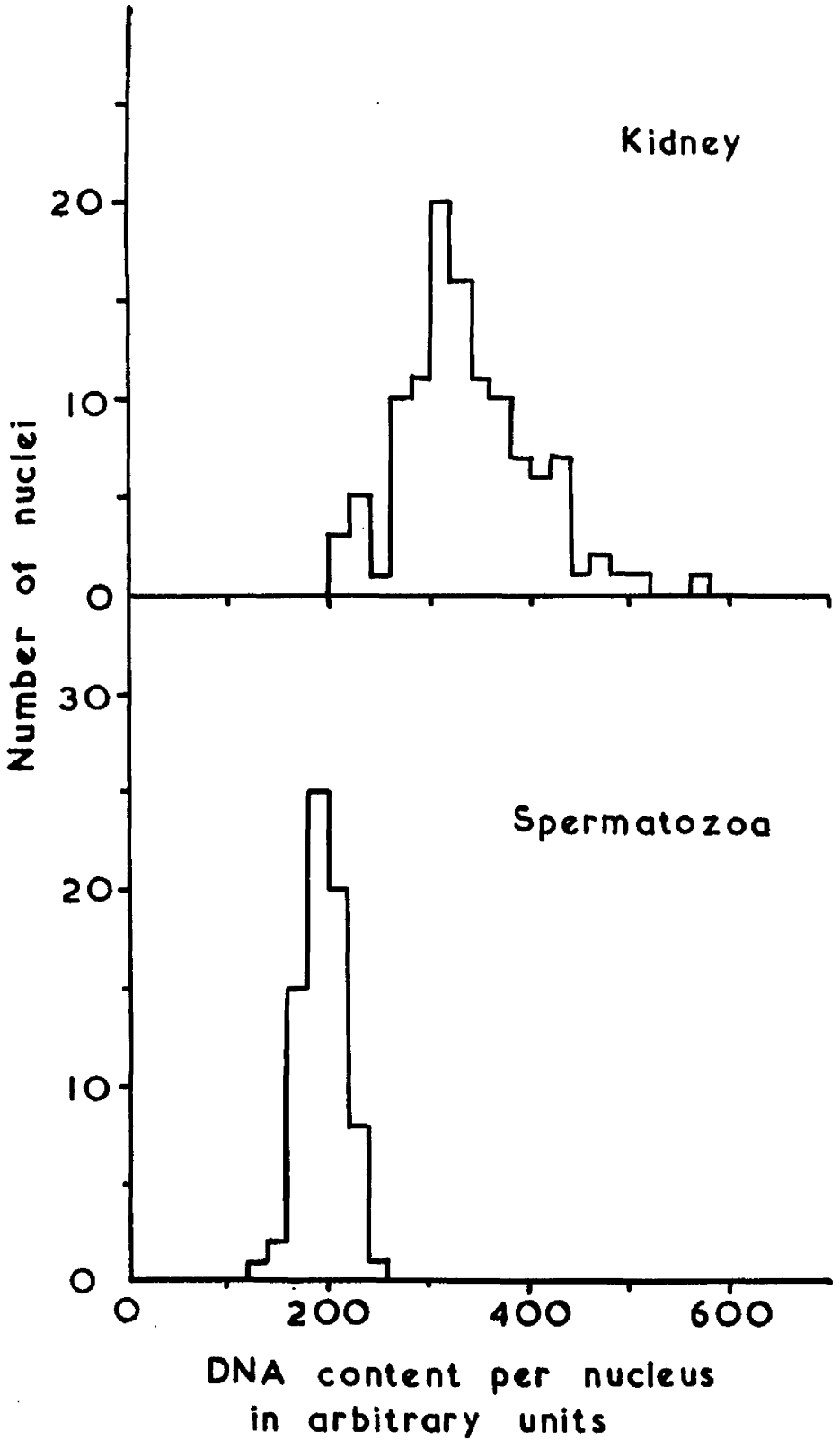
Kidney
20
10
0
Number of nuclei
30
Spermatozoa
20
10
0
0
200
400
600
DNA content per nucleus
in arbitrary units

Figure 13.

amount of DNA present and the state of the nucleus in relation to the mitotic cycle. Direct measurements on the same nucleus at different stages of its life-history are at present impracticable, since staining procedures inevitably interrupt the life-cycle, while ultraviolet measurements also interfere with normal cell processes through the effects of irradiation. Further, the nucleus undergoing visible mitosis is, from the optical point of view, unsuited to the photometric procedures used in the present experiments, since uniform dispersion of Feulgen-positive material in a roughly spherical nucleus cannot be achieved once chromosomes are visible. An alternative approach, however, is to study the overall picture of the DNA contents of a number of individual nuclei in a tissue which is known to be mitotically active, i.e., in a state of embryonic growth, active regeneration or hypertrophy, or the site of tumour growth. Conditions of active regeneration are readily obtainable in rat liver, by subjecting the animal to the operation of partial hepatectomy, and removing the liver remnant for study a few hours or days after operation, by which time the process of compensatory hyperplasia is under way. Conditions of tumour growth may also be readily produced in rat liver by the administration of the dye dimethylaminoazobenzene, the resulting hepatoma-bearing liver being used for isolation of nuclei as before.

The DNA contents of nuclei, isolated from the liver remnant removed from a rat, 48 hours after two-thirds of the liver had been extirpated, are shown in Figure 14. Again, control measurements on a group of kidney nuclei are presented for comparison. 139 kidney nuclei form the usual single peak, around a mean value of 557 units of DNA content, with the exception of one nucleus whose value of 1080 units suggests that it belongs to Class II. The nuclei from regenerating liver, however, present a very different aspect, with DNA contents ranging from 320 to 5200 units, a much wider range than in normal liver. If we subdivide these nuclei into classes, taking the upper limit of the Class I kidney nuclei (680 units) as the upper limit for liver Class I also, then we may classify the 371 liver nuclei measured into

214 Class I nuclei, 58% of the sample, mean DNA content 580 units

103 Class II nuclei, 28% of the sample, mean DNA content 1224 units

49 Class III nuclei, 13% of the sample, mean DNA content 2373 units

and 5 Class IV nuclei, 1.4% of the sample, mean DNA content 4508 units.

The mean DNA contents of the four classes of nuclei in the regenerating liver are in the approximate ratios 1:2:4:8, while the mean DNA content of Class I liver nuclei is again reasonably near to the mean value for Class I

Figure 14.

Frequency histograms of the relative deoxy-ribonucleic acid (DNA) contents of individual nuclei from rat kidney, and from rat liver regenerating after partial hepatectomy, as estimated by photometric measurements after Feulgen staining.

Class interval = 80 units.

(The arbitrary units of DNA content are applicable to this figure only.)

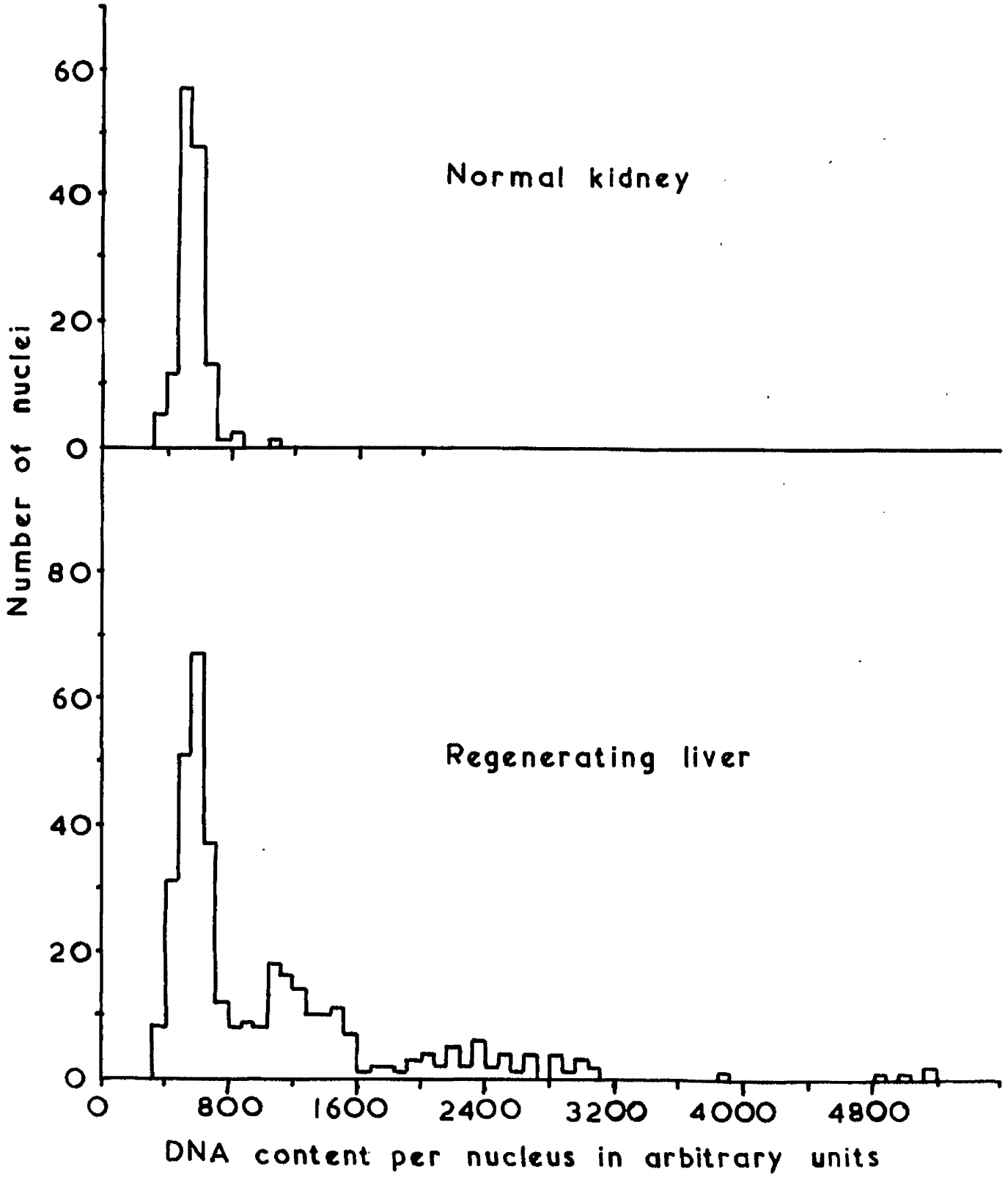
Normal kidney
Regenerating liver
Number of nuclei
60
40
20
0
80
60
40
20
0
0
800
1600
2400
3200
4000
4800
DNA content per nucleus in arbitrary units

Figure 14.

kidney nuclei. By comparison with normal liver, however, there has been a distinct fall in the proportion of Class II nuclei (from 40%, in the nuclei represented in Figure 8, to 28% in those in Figure 14) while there has been an almost exactly equivalent increase in the proportion of Class III nuclei. There is also to be noted the appearance of a small number of, presumably, Class IV nuclei, having approximately twice the DNA content of the already large Class III nuclei. This change in the proportions of the various classes of nuclei is almost certainly responsible for the temporary increase in the mean DNA content per nucleus reported for rat liver after partial hepatectomy. (Price and Laird, 1950; Thomson, Heagy, Hutchison and Davidson, 1953.)

Figure 15 shows the relative DNA contents of individual nuclei from rat kidney and tumour-bearing liver, derived from an animal maintained for 14 weeks on the hepatoma-producing diet described in Section 2.3a. As in previous experiments, the control kidney nuclei form one Class I group, the mean value in this experiment being 358 units, with the exception of two nuclei, with values of 700-800 units, which apparently belong to Class II. The great majority of the nuclei from the tumour-bearing liver fall into two groups, again corresponding to Class I and Class II, while a few with higher values may be regarded as belong-

Figure 15.

Frequency histograms of the relative deoxyribonucleic acid (DNA) contents of individual nuclei from rat kidney and from rat liver bearing hepatomas induced by administration of dimethylaminoazobenzene, the estimations being made by photometric measurements after Feulgen staining.

Class interval = 40 units.

(The units of DNA content are arbitrary, and are applicable to this figure only.)

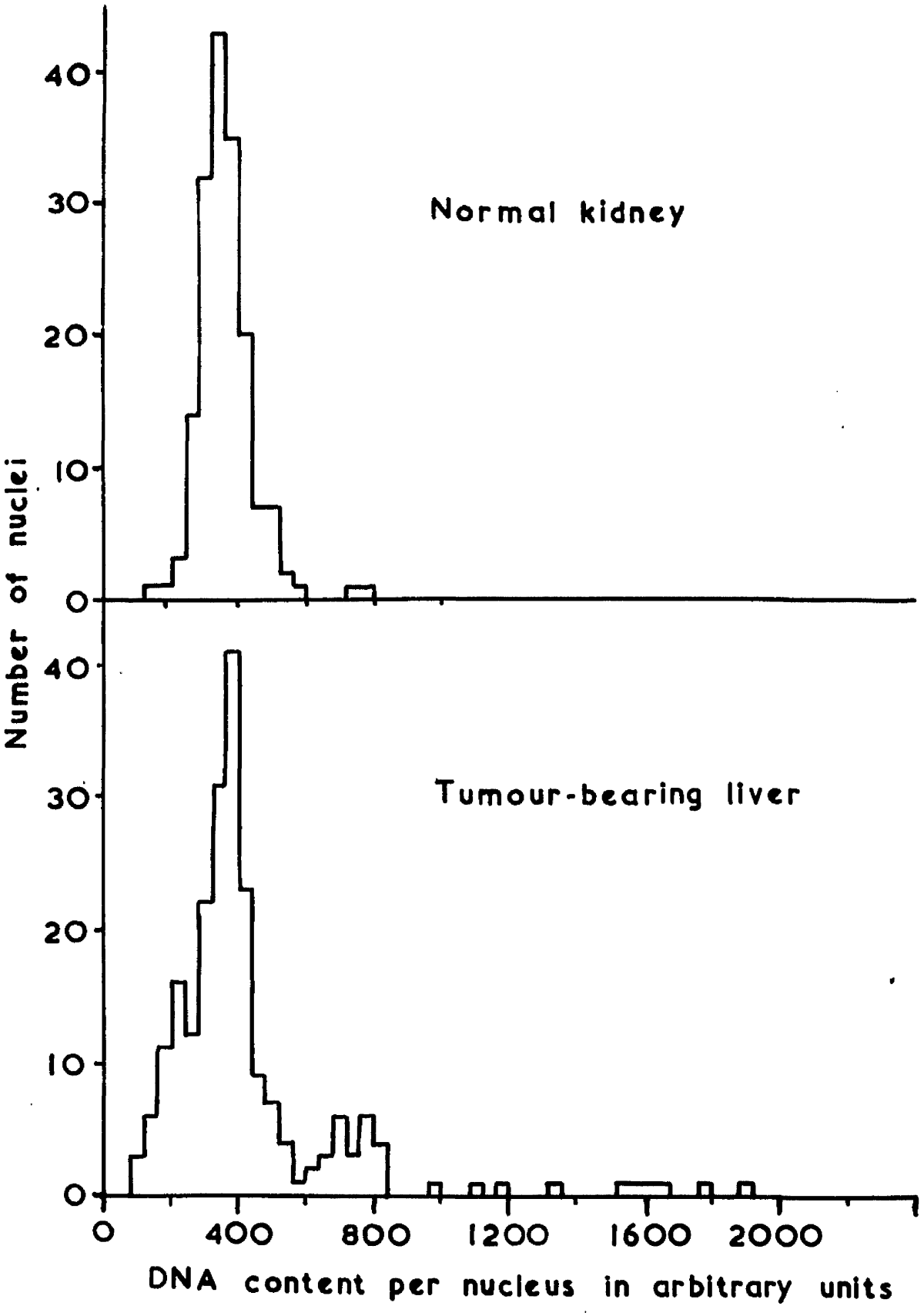
Normal kidney
Tumour-bearing liver
Number of nuclei
0
10
20
30
40
0
400
800
1200
1600
2000
DNA content per nucleus in arbitrary units

Figure 15.

ing to Class III. Two nuclei were found with values greatly in excess even of the Class III nuclei, and which have been provisionally ascribed to Class IV. The distribution of the 226 nuclei which were measured is as follows:-

186 Class I nuclei, 84% of the sample, mean value 336 units of DNA

24 Class II nuclei, 10% of the sample, mean value 733 units

10 Class III nuclei, 4.5% of the sample, mean value 1468 units

2 nuclei, possibly Class IV, 0.9% of the sample, mean value 3140 units.

The mean DNA contents of nuclei in the four groups again approximate to the ratio 1:2:4:8, but by comparison with normal liver the proportion of Class I nuclei to Class II nuclei has been very considerably increased. It is difficult to interpret the significance of this finding, since the tissue from which the nuclei were isolated consists of a variable amount of more or less normal liver tissue enclosing areas of disorganised overgrowth, the hepatoma nodules. One possible explanation for the change might be that normal liver cells, with mainly Class II nuclei, have been partially replaced by tumour cells having mainly Class I nuclei. It is also possible that when there is destruction of normal liver tissue by compression from the tumour tissue, the non-hepatocyte cells may prove to

be more resistant, and so come to represent a greater proportion of the cells present.

5.4. Results by Microspectrophotometry of Embryonic Chick Tissue Nuclei.

Figure 16 presents the results of a small series of measurements on nuclei from cultures of chick heart fibroblasts, and on nuclei from the liver of a chick embryo. The mean DNA content of nuclei in fowl tissues is considerably lower than that found for the rat nucleus (Mirsky and Ris, 1951; Davidson, Leslie, Smellie and Thomson, 1950) and consequently lower extinctions for Feulgen-stained material are to be expected.

In the fibroblast culture, 74 nuclei gave a mean value of 120 units of DNA, while 80 embryo liver nuclei gave a rather higher mean value, 145 units. In each case, the shape of the curve in the frequency histogram is asymmetrical, with a few values below the peak, and a number of nuclei have DNA contents approaching twice the peak value for the group. If we regard the peak frequency value as corresponding to the DNA content of an interphase nucleus having a diploid chromosome set, then the asymmetrical peaks are consistent with the presence of a considerable proportion of nuclei whose DNA content is approaching a level double that of the diploid nucleus. Such nuclei might

Figure 16.

Frequency histograms of the relative deoxyribonucleic acid (DNA) contents of individual nuclei from chick heart fibroblast cultures and from chick embryo liver, as estimated by photometric measurements after Feulgen staining.

Class interval = 20 units.

(The units of DNA content are arbitrary, and are applicable to this figure only.)

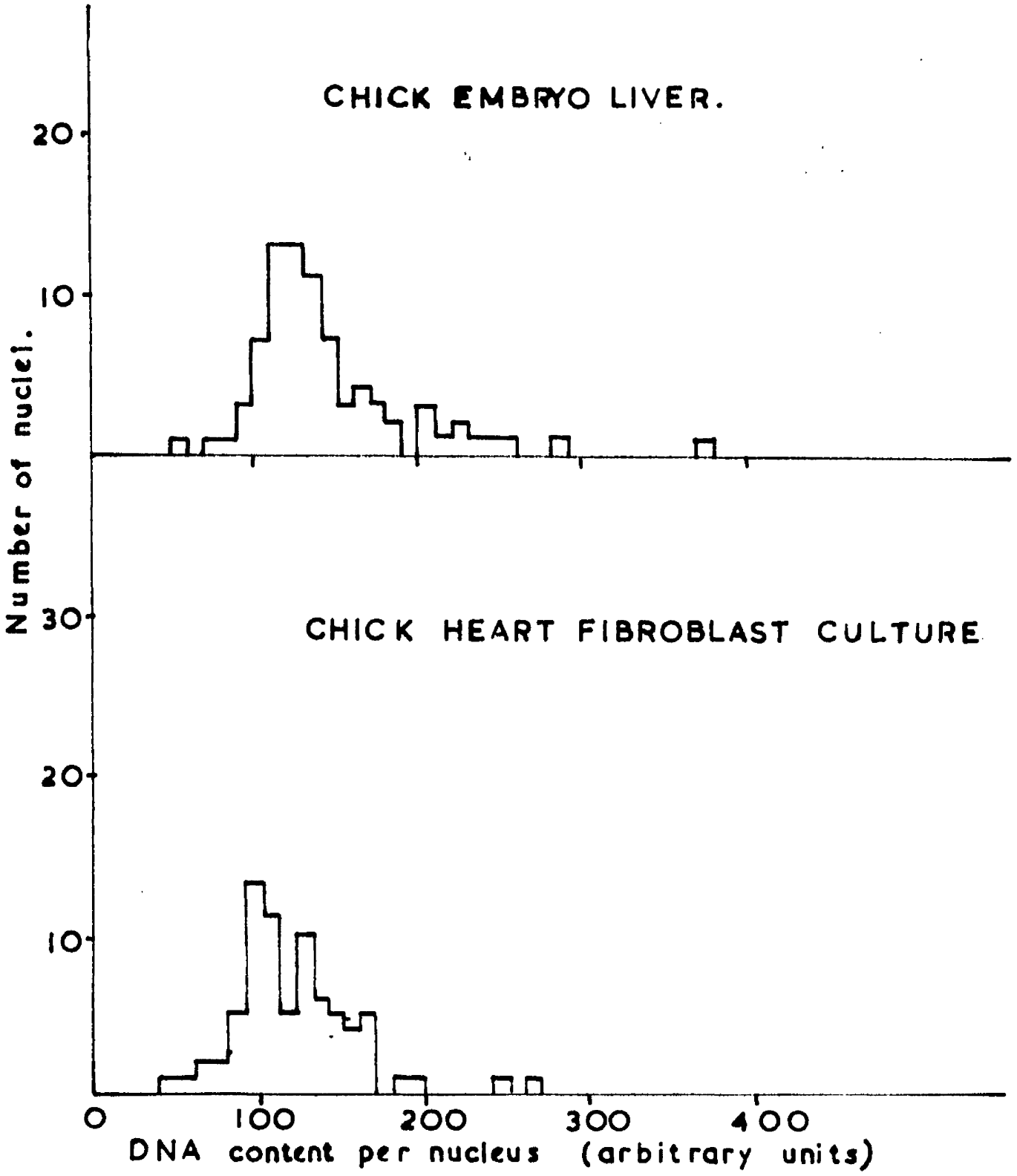
CHICK EMBRYO LIVER.
20
10
Number of nuclei.
30
CHICK HEART FIBROBLAST CULTURE
20
10
0
100
200
300
400
DNA content per nucleus (arbitrary units)

Figure 16.

reasonably be regarded as in the process of doubling their DNA content in preparation for visible mitosis.

The picture for liver, though based on comparatively few nuclei as compared with the earlier experiments on rat liver nuclei, shows indications of the presence of a small Class II peak, or at least of a proportion of nuclei whose DNA content is twice that of the peak value for the majority of the nuclei. Such nuclei, however, are few, by comparison with the proportion regularly found in rat liver. One nucleus was found which appears to belong to Class III.

3.5. Statistical Assessment of Differences in DNA Content of Nuclei a) in Rat Liver, and b) in Rat Kidney.

Although in a tissue such as liver the nuclei may be fairly readily classified, according to their DNA content, into three or more groups, whose mean values approximate reasonably closely to the ratios 1:2:4:8, it is also clear from the frequency histograms that within each group there exists a considerable scatter of values, so much so, in several cases, that it is difficult to decide where one class ends and the next begins. In the group of kidney nuclei whose DNA contents are shown in Figure 8, for example, the values within the apparent Class I peak range from 280 to 720 units, around a mean value of 500 units, with a standard deviation of 87 units. The coefficient of

variation for this group is therefore 87/500 x 100, i.e., 17.4%. Although one might be tempted to regard this scatter as arising from the errors and assumptions inevitably connected with the present photometric technique, it is most desirable to try to determine whether all of this scatter of values is reasonably attributable to experimental errors, or whether part of it is due to true biological variation between one nucleus and another. At the one extreme, it might be found that the photometric estimations possessed a high degree of accuracy and reproducibility, so that the differences between the observed values arose almost entirely from true differences between the nuclei. At the other extreme, all of the scatter of values might arise from experimental inaccuracies attributable either to the observer or to the deficiencies of the apparatus and technical methods, with no significant difference between individual nuclei within a group. One possible method of investigating the significance of the scatter within each group is to carry out replicate measurements on a number of nuclei, and to submit the values obtained to analysis of variance (Snedecor, 1946; Brownlee, 1948). By this means, it should be possible to arrive at a numerical assessment of the true differences, if any, between the nuclei.

The procedure adopted was as follows:- 24 Feulgen-stained kidney nuclei contained in one satisfactorily exposed negative were numbered. The DNA contents of these nuclei were then estimated in the normal manner, four times each, the nuclei being measured in random order each time (the random sequence being taken from tables of random numbers). Table 3 contains the data obtained. Table 4 presents the analysis of variance of the DNA contents of the 24 nuclei detailed in Table 3, and indicates that there is in fact a significant difference in DNA content between the kidney nuclei, the coefficient of variation attributable to this cause being 4.8%. In approximate terms, then, one can say that three-quarters of the scatter of values found for the DNA content of kidney nuclei is due to experimental error, and one quarter to true variation between the kidney nuclei. Even this estimate of the true variation between the nuclei may be somewhat high, since all errors in the measurements cannot be estimated through replicate measurements, e.g., the error arising from inhomogeneous distribution of stained material within the nucleus being measured.

A similar *ad hoc* experiment was carried out on Feulgen-stained liver nuclei, the results being presented in Tables 5, 6 and 7. 59 nuclei contained in three consecutive frames of a film strip were each measured twice with respect to their DNA content, the order of the

Table 3.

Statistical assessment of the significance of the differences in DNA content between individual rat kidney nuclei.

DNA contents, by Feulgen method, of 24 kidney nuclei measured four times, in random order each time.

Nucleus No.	DNA content of nucleus (arbitrary units)			
	First Measurement	Second Measurement	Third Measurement	Fourth Measurement
1	455	412	361	381
2	445	382	438	330
3	346	322	347	322
4	378	332	371	335
5	343	315	400	375
6	377	375	426	376
7	374	382	332	312
8	437	422	469	346
9	349	347	351	306
10	374	402	400	345
11	352	376	340	340
12	386	347	348	376
13	346	381	358	375
14	324	319	368	373
15	383	372	420	349
16	395	336	354	420
17	311	365	378	314
18	374	339	312	334
19	467	333	376	377
20	339	356	402	349
21	460	363	393	333
22	328	326	288	331
23	340	460	341	323
24	384	339	368	335

Grand mean for 24 nuclei = 365.3 units of DNA
For analysis of variance, see Table 4.

Table 4.

Statistical assessment of the significance of the differences in DNA content between individual rat kidney nuclei.

(Analysis of variance of measurements listed in Table 3.)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio "F"
Between nuclei	23	54,537	2,371.2	2.09
Between estimates	3	12,197	4,065.7	
Residual error	69	78,197	1,133.3	
Total	95	144,931		

For $n_1 = 24$, $n_2 = 70$, $F = 2.07$ on the 1% significant level.

There is therefore a significant difference in DNA content between the individual nuclei.

The component of the variance corresponding to the difference is $\frac{2,371.2 - 1,133.3}{4} = 309.5$.

Since the grand mean for the 24 nuclei = 365.3, the coefficient of variation between nuclei

$= \frac{\sqrt{309.5}}{365.3} = 4.8$ per cent.

Table 5.

Statistical assessment of the significance of the differences in DNA content between individual rat liver nuclei.

DNA contents, by Feulgen method, of 59 liver nuclei measured twice, in random order each time. The nuclei are listed in Classes I and II according to the mean of the two estimates, taking 600 units as the boundary between the classes (see Figure 17 for frequency histogram).

Class I nuclei

Nucleus No.	DNA content of nucleus (arbitrary units)	
	First Measurement	Second Measurement
1/3	460	462
1/4	396	522
1/5	430	510
1/7	485	352
1/10	264	280
1/12	309	281
1/14	374	297
1/15	429	277
1/16	388	371
2/1	422	424
2/3	435	477
2/4	539	551
2/6	275	271
2/7	340	367
2/8	205	234
2/9	482	506
2/12	315	399
2/13	345	354
2/15	315	346
2/16	531	409
2/17	347	436
2/20	375	357
2/21	424	413
3/1	304	286
3/3	306	292
3/5	276	281
3/6	292	283
3/7	413	423
3/8	357	355
3/10	373	316
3/12	404	297

Table 5 (Contd.)

Class I nuclei (contd.)

Nucleus No.	DNA content of nucleus (arbitrary units)	
	First Measurement	Second Measurement
3/13	320	405
3/15	314	255
3/16	348	367
3/18	409	372
3/19	413	420

Grand mean for 36 Class I nuclei = 370.3 units of DNA.

Table 5 (Contd.)

Class II nuclei.

Nucleus No.	DNA content of nucleus (arbitrary units	
	First Measurement	Second Measurement
1/1	834	600
1/2	794	875
1/6	924	910
1/9	846	831
1/11	1042	1037
1/13	964	945
1/17	939	1015
1/18	835	882
1/19	1118	971
2/2	995	974
2/5	889	680
2/10	1042	977
2/14	952	1141
2/18	788	685
2/19	1090	1053
2/22	940	885
2/23	746	913
3/2	687	720
3/4	695	836
3/9	1019	1044
3/11	1045	996
3/14	785	1008
3/17	898	1040

Grand mean for 23 Class I nuclei = 910.5 units.

For analysis of variance, see Tables 6 and 7.

Table 6.

Statistical assessment of the significance of the differences in DNA content between individual rat liver nuclei belonging to Class I.

(Analysis of variance of measurements on Class I nuclei listed in Table 5.)

Source of variation	Degree of freedom	Sum of squares	Mean square	Variance ratio "F"
Between nuclei	35	376,694	10,763	5.669
Between estimates	1	382	382	
Residual error	35	66,451	1,899	
Total	71	443,527		

For $n_1 = 30$, $n_2 = 34$, $F = 2.30$ on the 1% significance level.

There is therefore a significant difference in DNA content between the individual nuclei.

The component of the variance corresponding to this difference is $\frac{10,763 - 1,899}{2} = 4,432$.

Since the grand mean for the 36 nuclei was 370.3, the coefficient of variation between nuclei = $\frac{\sqrt{4,432}}{370.3} = 18$ per cent.

Table 7.

Statistical assessment of the significance of the differences in DNA content between individual rat liver nuclei belonging to Class II.

(Analysis of variance of measurements on Class II nuclei listed in Table 5.)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio "F"
Between nuclei	22	597,496	27,158	3,835
Between estimates	1	495	495	
Residual	22	155,788	7,081	
Total	45	753,779		

For $n_1 = 24$, $n_2 = 22$, $F = 2,75$ on the 1% significance level.

There is therefore a significant difference in DNA content between the individual nuclei.

The component of the variance corresponding to this difference is $\frac{27,158 - 7,081}{2} = 10,039$.

Since the grand mean for the 23 nuclei = 910,5, the coefficient of variation between nuclei $= \frac{\sqrt{10,039}}{910,5} = 11$ per cent.

measurements of extinction and area being random each time. Table 5 contains the numerical data obtained, while Figure 17 is the frequency histogram of the means of the two values for each nucleus. The usual Class I and Class II peaks are present, and in Table 5 the values for the nuclei within the two distinct peaks are listed separately. Tables 6 and 7 contain the analyses of variance for the Class I and Class II liver nuclei respectively. In both cases, there is a significant difference between the DNA contents of the individual nuclei within the two classes (as well as the obviously significant difference between members of Class I and Class II), the true coefficient of variation between Class I liver nuclei being 18% of the mean, and 11% of the mean for Class II. The fact that these coefficients for liver nuclei are considerably greater than that for kidney nuclei need not necessarily be entirely due to a greater biological variability between the DNA contents of individual liver nuclei. It might, for example, be due to the less uniform distribution of Feulgen stain occasionally seen in liver nuclei as compared with kidney nuclei, a source of error which cannot be eliminated by the replication of measurements on a single photographic negative.

Figure 17.

Frequency histograms of the relative deoxyribonucleic acid (DNA) contents of individual rat liver nuclei, as estimated by photometric measurements after Feulgen staining.

Class interval = 80 units.

Each value is the mean of the two values recorded in Table 5.

Class I nuclei have values from 0 - 600 units.

Class II nuclei have values from 601 - 1200 units.

(The arbitrary units of DNA content are the same as those recorded in Table 5, but are not comparable with the other figures.)

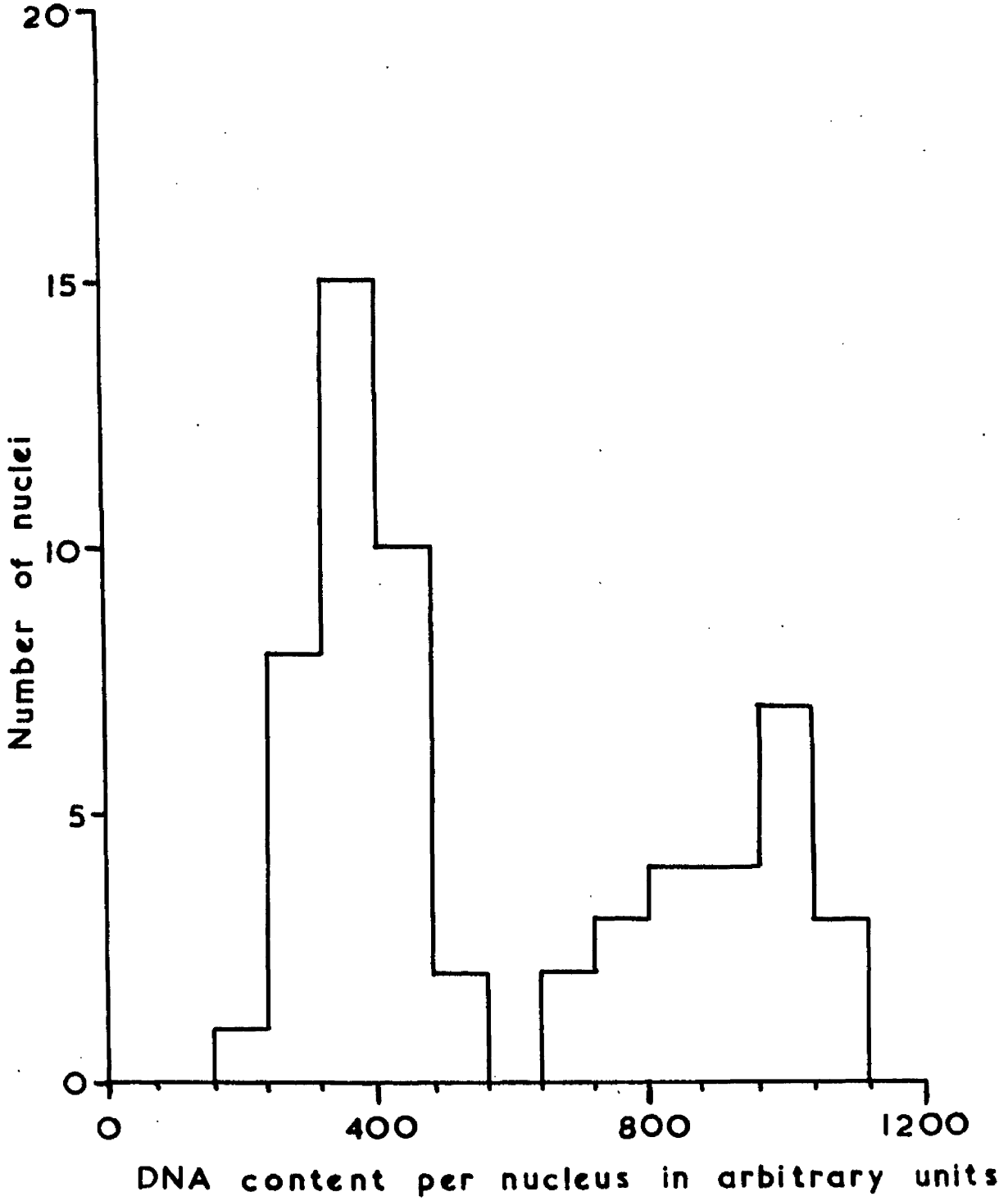
20
15
10
5
0
Number of nuclei
0
400
800
1200
DNA content per nucleus in arbitrary units

Figure 17.

PART IV.

DISCUSSION.

Discussion.

4.1. The validity of microspectrophotometry as a tool for the chemical study of the cell nucleus.

The principal barrier to the chemical study of the single cell lies in the extremely minute amounts present of even its major constituents, so that direct chemical analysis is, by orthodox methods, quite impossible. An alternative approach, and one which has been widely exploited, is to analyse a large number of cells or cell components, and to count, by dilution and sampling, the actual number of units present. In this way it is easy to calculate the amount of a particular constituent present in, say, a single nucleus, but the figure obtained is of course only a mean value and does not take into consideration the variation between individual nuclei. In cases where the tissue studied consists of one cell type only, e.g., erythrocytes in the adult, the variation in composition from cell to cell may be slight, but within most tissues it is at least probable that considerable variations between cells do occur. Ingenious ultramicro-analytical methods have been devised, which in special cases may permit the direct analysis of one cell for a few constituents, e.g., the microionophoretic technique of Edstrom (1953) for the separation and estimation of the ribonucleotides contained in a single nerve cell,

but such methods are useful in special circumstances only. Since the microscope can produce a visible and reliable image of an object far beyond the resolving power of the human eye, and since small amounts of many substances can be accurately measured by means of their characteristic optical properties, it is logical to combine these two aids to the senses in the form of a microspectrophotometric instrument, whose aim is to measure the light absorption properties of very minute amounts of material, e.g., the amounts present in single cells. The necessary basic information is usually obtainable on a macro-scale, namely, the light absorption spectra of substances to be measured, their specific or molecular extinction coefficients or, in cases where some colour-producing reaction is to be used, the relationship between concentration of the unknown and colour intensity produced. If one then introduces a microscope as part of the optical train of an otherwise conventional colorimetric instrument, valid spectrophotometric measurements may be obtained on a micro-sample of material, provided, firstly, that the distribution of light intensity in the image plane of the microscope corresponds to that in the object plane, and secondly, that the loss of light in the specimen occurs by absorption only.

The first of these requirements has been checked by making measurements on suitable test objects, e.g.,

coloured glass plates whose density can be measured on a macro-scale for comparison, suspensions of spherical droplets of coloured oil, dyed cellulose acetate films, etc., and workers who have measured such objects report good agreement between the results given by the microspectrophotometer and those found by the normal spectrophotometer (Ris and Mirsky, 1949; Pollister and Swift, 1950). The second requirement, that of negligible loss of light other than through absorption by molecules of the solute, appears at first sight to be impossible of attainment in a specimen consisting of a complex colloidal system which has been precipitated and dehydrated. Caspersson, however, has studied the matter from both theoretical and practical aspects (Caspersson, 1940, 1950b), and concludes that careful choice of optical conditions can permit of measurements with a reasonable degree of accuracy, and especially if the medium in which the specimen is mounted is one which permits of even a small degree of swelling of the colloidal particles. Other workers have adopted an empirical approach to the question of non-specific light absorption, by making direct measurements of light absorption on the unstained specimens whose subsequent intensity of staining is to be measured by the microspectrophotometer. Korson (1951) and Ris and Mirsky (1949) have found the extent of non-specific light absorption by cell nuclei, in the visible region of the spectrum at least,

to be so low that it could be neglected altogether in comparison with other errors. (The same may not necessarily be true outside the visible spectrum.) Provided measurements of optical density are made on homogeneous spherical objects in a medium of suitable refractive index and provided the measurements are made close to the centre of the sphere, the extent of the error due to diffraction, even in the U-V region, is small. Caspersson (1940), however, regards an optical density of 0.02 as the lower limit of accurate measurement. (In the experiments reported in this series, the lowest recorded optical densities were of the order of 0.15, while the great majority were in the region of 0.30-0.50.).

Thus from the purely instrumental point of view, the microspectrophotometer, devised by Caspersson and subsequently exploited by many other workers, can be regarded as capable of providing reasonably reliable data on the optical density of small objects, if the necessary optical conditions are maintained in the instrument and in the object measured. It is in assessing the significance of such measurements that there is room for dispute, since not all workers are agreed that the commonly used microspectrophotometric procedures do in fact measure what their protagonists claim.

As regards ultraviolet absorption measurements,

Caspersson has claimed to be able to measure the absorption spectra of cell structures, with an adequate degree of precision for qualitative identification and location of certain classes of ultraviolet-absorbing substances, notably the nucleic acids and those proteins rich in cyclic amino acids. He has also claimed, by analysis of the absorption curves, or in some cases by analysis of absorption measurements made at three selected wavelengths in the ultraviolet region, to be able to calculate the amounts of protein and of mixed nucleotides, in terms of weights per square micron of tissue section (Caspersson, 1950b). Few would dispute the validity of the ultraviolet microspectrophotometric technique as a means of recording absorption spectra, while comparative quantitative measurements on objects such as cell nuclei are not likely to be greatly in error. It is when calculations of absolute amounts of material contained in precipitated colloidal systems are based on the extinction coefficients of the same material in bulk dilute solution, that the microphotometric results are of less certain validity. If it were possible to check, in the U-V region, the validity of the Beer-Lambert laws under the optical conditions prevailing in the typical cytophotometric specimen, the full validity of ultraviolet microspectrophotometry would be established. It appears safer at present to confine such studies to comparisons of the U-V absorptions

of different objects rather than to calculate absolute values therefrom.

In the present experiments, ultraviolet absorption measurements on rat cell nuclei were made at one wavelength only, 2536 A, which is slightly lower than that at which the maximum absorption of light by the nucleic acids is found, but is near to the wavelength of minimum ultraviolet light absorption by the proteins. Having regard to the much higher specific extinctions of nucleic acids as compared with proteins at this wavelength, and to their relative concentrations in the cell nuclei, one may regard the extent of ultraviolet light absorption by nuclei at 2536 A as being closely related to the amounts of nucleic acids or nucleotides which they contain. If, as Pollister (1952) has suggested, we regard the fixed and washed cell nucleus as containing little or nothing apart from protein and nucleic acids, then the absorption at 2536 A is a reasonably good measure of the amount of nucleic acid, both DNA and RNA, which that nucleus contains.

The present experiments have shown that the extent of absorption of U-V light by the rat cell nucleus can be considerably reduced by prior treatment with a solution of crystalline ribonuclease, and chemical experiments on similarly treated nuclei in bulk have also shown a very marked reduction in the amount of ribonucleic acid which they contain.

Thus the view of Leuchtenberger, Leuchtenberger, Vendrely and Vendrely (1952) that the influence of nuclear RNA in ultraviolet absorption measurements is negligibly small, is not supported by the present observations. The resulting measurements of ultraviolet light absorption, however, after removal of RNA, should form a basis for comparison with the amounts of DNA found by other methods.

The largely theoretical criticisms of Commoner (1949, 1950), that microspectrophotometric measurements of ultraviolet light absorption may be seriously in error on account of molecular orientation within the cell structures studied, do not appear to have any experimental backing. Commoner's criticisms have been answered by Pollister and Swift (1950) and by Caspersson and Schultz (1951), and unless some further evidence is forthcoming to substantiate the postulated errors, they may be assumed to be negligible as far as comparative measurements are concerned.

The validity of microspectrophotometric methods for the measurement of DNA, based on staining by the Feulgen method, have been more seriously criticised than have the ultraviolet absorption measurements of Caspersson. Part of the criticism arises from the as yet uncertain chemical basis of the reaction, although, as has already been discussed, it appears very likely that acid hydrolysis liberates aldehyde groupings in the DNA molecule, the residue of which still

remains insoluble in an acid medium. Also, a positive reaction may not necessarily be entirely due to DNA, since other aldehydes already present or liberated by the acid hydrolysis would presumably also re-colourise the Feulgen reagent. Such aldehydes would, of course, require to be present in an acid-insoluble form, otherwise they would be removed from the cell nucleus during the acid hydrolysis, or during the subsequent rinsing and washing. In any case, the prompt conversion of nuclei from Feulgen-positive to Feulgen-negative by treatment with deoxyribonuclease is strong evidence that a positive reaction is due to the presence of DNA. As a further check, one should also carry out the staining reaction without prior hydrolysis, when non-specific staining becomes apparent. No evidence of interference by other aldehydes was found in the present series.

In the important matter of whether the true location of DNA is revealed by Feulgen staining, the views of Stedman and Stedman on the role of "chromosomin" are well known (Stedman and Stedman, 1943, 1947, 1950). While it is probably true that the acidic proteins of the nucleus can be stained by basic dyes such as regenerated basic fuchsin, it has recently been shown that a positive Feulgen reaction can be obtained using a reagent consisting of bleached acid fuchsin in lieu of the usual basic dye (Kasten, 1955). This finding is consistent with the theory of the Schiff reaction

proposed by Wieland and Scheuing (1921), and applied to the Feulgen reaction by Lessler (1951), but is very difficult to explain on the basis of the Stedmans' view that chromosomin, and not DNA, is the material which is stained. Furthermore, kryptic digestion of a tissue section, which can hardly fail to have an effect on the amount of chromosomin present, has virtually no effect on the intensity of the Feulgen reaction, up to the time of disintegration of the specimen (Catcheside and Holmes, 1947). Callan (1943) has summarised the main criticisms of the Stedmans' views on chromosomin.

The divergent views of various investigators on the influence of the nature and duration of fixation, and the conditions and duration of hydrolysis and staining, on the final intensity of the Feulgen colour, all serve to emphasise that it is unwise to attempt to establish absolute values for DNA on the basis of even a standardised Feulgen staining technique unless every relevant variable can be rigorously controlled. That this is not easy is seen from some of the experimental results here reported. Samples of rat kidney nuclei, Feulgen-stained by apparently the same procedure in every case, but at different times, give mean values for their DNA contents in arbitrary Feulgen units which differ appreciably from one experiment to another. Yet samples stained simultaneously, on the same slide, give

mean values, for the Class I peaks in liver and kidney nuclei, which differ only slightly. In the present results, therefore, it has been strongly emphasised that direct comparison of the units of DNA content from one experiment to another is not justified. The standard sample of rat kidney nuclei included on each slide for comparison with the nuclei of the tissue being studied is the only valid link between the values reported for DNA content in the different experiments, and, in effect, serves as a side-by-side control experiment each time.

Even within a single experiment of this sort, it has not been reliably established that the amount of Feulgen dye retained in a nucleus bears a linear relationship to the amount of DNA which it contained. The check experiments of Lessler (1951) on DNA-gelatin drops indicated a linear relationship between Feulgen colour intensity and DNA concentration up to only 1 mg. of DNA per ml., a value very considerably below that found in the rat cell nucleus. Swift (1955) on the other hand, in analysing his data on mouse nuclei having varying degrees of ploidy, considers that the DNA-Feulgen interaction product obeys the Beer-Lambert laws up to a concentration of DNA equivalent to 0.3 g. in 1 ml. This calculation, however, assumes that a tetraploid nucleus contains twice the amount of DNA found in a diploid nucleus; the assumption may well be true, but

cannot be regarded as a fully satisfactory verification of the validity of calculations based on measurements of Feulgen dye intensity. This is especially true when one wishes to make use of the Feulgen technique to compare the amounts of DNA contained in diploid and tetraploid nuclei within a tissue.

Since it is impossible to prepare fully valid test objects whose DNA content is known, and in which the physicochemical conditions are the same as those found in the nuclei which one wishes to measure, the only alternative check procedure is an empirical one - to make as many measurements as possible on nuclei whose mean DNA content is known from chemical measurements, and to compare the results with those from other nuclei known to differ in mean DNA content. Ris and Mirsky (1949) and Leuchtenberger, Vendrely and Vendrely (1951), on the basis of such measurements on nuclei from a number of animal tissues, conclude that Feulgen measurements are in fact a valid basis for study of amounts of DNA per nucleus. However, such criteria of validity as have been applied to microspectrophotometric techniques are considerably less rigorous than those customarily applied when verifying experimental methods. In the writer's opinion, it is not permissible to invest any microspectrophotometric measurement with more than relative significance, and where the measurement is based on a staining reaction

whose precise stoichiometry is not known (as is the case with the Feulgen reaction) it is unwise even to compare measurements made on material stained by the same technique but at different times, unless some common basis for comparison can be found. In the present experiments, such a standard has been used in the form of a preparation of normal rat kidney nuclei, whose DNA content, both by chemical estimations and by microspectrophotometry, shows reasonably small variation.

The question of how far the variation between the DNA contents of single nuclei is a true biological variation and how far it is attributable to experimental error has hitherto been somewhat neglected, most workers merely reporting mean values for groups of nuclei, with in some cases estimates of the standard error. Descriptions of photometric apparatus have commonly contained statements that the readings were reproducible to within a specified percentage, without, however, indicating the extent of errors arising from causes other than those inherent in the instrument. Part of the difficulty in assessing the significance of variations between nuclei has arisen from the comparatively small numbers of nuclei which can conveniently be measured, so that adequate statistical study of the variations is not easy. In the present experiments, one series of measurements has been made with the express

purpose of studying the extent of the experimental error, as shown by replicate measurements on the same nuclei. Such analysis does not cover the entire range of possible errors, but is confined to the errors of measurement, including observer error -- it does not, for example, allow for inaccuracies arising from the methods of preparation of the material for study. The results of co-variance analysis of measurements made on a batch of kidney nuclei indicate that there is, within the group, a coefficient of variation of about 17%, of which only about a quarter is due to true variation between the nuclei, the remainder being due to experimental errors. Although no detailed statistical inquiry, to determine the source of these errors, was possible in the present series, the opinion of both workers who have used the method is that the determination of the sizes of nuclei (in units of area measured by planimeter on enlarged outlines of each nucleus) is liable to considerable observer error, while the measurement of extinctions is more satisfactory. Pollister and his co-workers (Pollister and Ris, 1947; Leuchtenberger, 1950) measure nuclear sizes by enclosing the approximately circular nuclear image in the aperture of a calibrated iris diaphragm, a method likely to give rise to serious errors also. Measurements by eye-piece micrometer are hardly likely to be any more satisfactory, especially with non-spherical nuclei, although to

the observer such measurements may appear to have a degree of precision which is largely illusory.

Inhomogeneous distribution of absorbing material within the nucleus is a possible source of serious error, particularly in fixed specimens, unless steps are taken to ensure even precipitation of the nucleoproteins before staining or U-V measurement. Admittedly it is, in theory, possible to scan a single nucleus completely by a suitably small spot of light, and to arrange for integration of the photocell's output during the scan. The same effect can be achieved by means of photographic photometry, if the negative of the nuclear image is scanned completely by a recording densitometer, and apparatus for this purpose has been developed (Caspersson, Jacobsen and Lomakka, 1951; Walker and Yates, 1952a, b). Such total image scanning, however, involves the measurement of the apparent extinction at the periphery of the nuclear image, in which region the non-specific light loss by scattering and diffraction will be at its maximum relative to the light loss by specific absorption, and unless the refractive index of the mounting medium has been very carefully matched to that of the light-absorbing material within the nucleus, the extent of the error may be considerable. It is preferable to obtain homogeneous dispersion of the light-absorbing material, and to confine the absorption measurements to the central zone

of the nucleus, where the error in extinction measurements, caused by refractive index differences, is small. Fortunately, treatment with sucrose prior to fixation (or prior to nuclear isolation in citric acid) secures good dispersion of the "chromatin" throughout the nucleus, and inhomogeneity errors can be very greatly reduced, though by no means totally eliminated.

Thus we see that results obtained by the technique of microspectrophotometry, as applied to biological specimens such as cell nuclei, should still be interpreted with very great caution. Indeed, at the present time the justification for its use lies in the total lack of other methods for quantitative study of the chemical composition of the single cell nucleus. The application of microspectrophotometric techniques, with due regard to their limitations, and with the use of all available means to verify their validity in particular cases, should undoubtedly play a useful part in establishing the role of the cell nucleus in the processes of life, even although the standard of accuracy obtainable is lower than one would wish.

4.2. The relative merits of the ultraviolet, Feulgen and methyl green techniques for measurement of deoxyribonucleic acid.

The three methods which have been used, in the present experiments, for estimating the DNA content of single

nuclei, though all depending on microspectrophotometric measurements, differ in their physical or chemical basis. Thus the measurements of ultraviolet light absorption at around 2600 A are primarily dependent on the absorption of light by cyclic compounds having conjugated double bond systems, the purine and pyrimidine bases being the relevant components of the nucleic acids. Feulgen staining, on the other hand, is apparently due to the aldehyde groupings liberated by mild acid hydrolysis of the deoxypentose sugar residues in the molecule of DNA, while staining with the basic dye methyl green is primarily due to the presence, in DNA, of phosphoric acid residues. Although each class of measurement has been criticised on account of the possibility of interference by other compounds, known or unknown, it was considered at the outset of the experiments here reported that some information on the validity of the methods could be obtained by carrying out a series of measurements, on nuclei from the same batches, by all three techniques. This has been done for nuclei from rat liver and from rat kidney, although the units of DNA content found by each technique necessarily differ and cannot be directly compared. It can be seen, however, from the patterns of DNA content shown in the frequency histograms obtained by the three methods, that there is a striking resemblance between the three sets of results. Kidney nuclei in each case fall

into one group with regard to the amounts of Feulgen or methyl green dye which they take up, and the same is true for ultraviolet absorption measurements if the influence of nuclear RNA is eliminated by prior treatment with ribonuclease. Liver nuclei form two major groups, one having double the content of dye, or double the extent of U.V light absorption, of the other, while there is a small third group with four times the value of the lowest group. Also, the mean values for the kidney nuclei by all three methods correspond closely to the mean values found for the lowest liver group. Although this cannot be taken as rigorous proof of the validity of the methods, it is, at the least, strongly suggestive that all three techniques are measuring essentially the same substance, and particularly when the differing chemical basis of the three methods is considered.

Since the application of microspectrophotometric techniques to any considerable number of nuclei is laborious, it was considered impracticable to continue to use all three methods for the study of nuclei from tissues other than rat liver and kidney, and therefore the merits and demerits of each technique had to be considered.

1). Ultraviolet microspectrophotometry.

Ultraviolet microscopy using refracting objectives is technically difficult, particularly as regards

achieving good focus in the image. Since final focussing may require several photographic exposures, a considerable amount of time is required to secure acceptable ultraviolet pictures of even a few nuclei. If a photoelectric technique is employed for U-V cytophotometry, then not only is there the same difficulty in ensuring satisfactory focus, without which photometric measurements are liable to serious error, but the accurate centering of the image of a nucleus on to the photomultiplier cathode is also difficult. Having only a feeble and poorly-defined visible image on the fluorescent screen, the observer is at some disadvantage when he wishes to identify individual nuclei within a field, while accurate measurement of nuclear size is almost impossible except on photographic plates. For these reasons, the photographic technique of photometry appears specially suitable for ultraviolet work. It also has the considerable advantage that it is possible to repeat all the measurements if desired, whereas photoelectric measurements could only be repeated if the nucleus could be re-identified.

Ultraviolet microspectrophotometry, alone among the three techniques, rests on a firm physico-chemical basis, and is independent of the uncertainties in stoichiometry which beset the visible-light techniques. On the other hand, the measurements are liable to interference from substances other than DNA, though their effect can be

considerably reduced by suitable methods of preparation of the specimen for microscopy.

2). Cytophotometry following methyl green staining.

The validity of the methyl green technique depends on the work of Kurnick and others in investigating the specificity and stoichiometry of the methyl green reaction for DNA (Kurnick, 1950; Kurnick and Foster, 1950; Pollister and Leuchtenberger, 1949; Vercauteren, 1950). Both Kurnick and Vercauteren consider that ability to stain with methyl green is related to the state of polymerisation of the DNA molecules, although Taft (1951) disagrees with their conclusions. In the present series of experiments, it was found that the methods for preparing and staining the specimens, as published by Kurnick, gave staining so faint as to be negligible, and certainly quite unsuited to photometric measurement. The same result was found in all experiments using the technique advocated by Kurnick, although his published methods were followed in every detail. Whatever the cause of the difficulty may be, the satisfactory staining achieved by the method of Pollister (1950) suggests that other factors than the molecular size of DNA are important in determining the extent of methyl green staining, a conclusion which arouses some misgivings regarding the stoichiometry of the interaction between methyl green and DNA.

By contrast with the ultraviolet methods, cytophotometry following methyl green staining is technically straightforward, and reasonable numbers of nuclei can be measured fairly quickly. (Care must of course be taken to verify that the methyl green used is true hexa- or heptamethylpararosaniline, as the other so-called methyl green stains available have no specific affinity for DNA.)

3). Cytophotometry following Feulgen staining.

The practical advantages of Feulgen staining for cytophotometry, as compared with the ultraviolet method, are considerable. Selection, focussing and centering of the specimen are rapid and reliable, while if a photoelectric instrument is to be used, standard glass-envelope photocells can be employed. The peak of light absorption by Feulgen stained material is close to the 546 mμ "mercury green" line, which is a convenient source of monochromatic light for Feulgen photometric measurements. The problem of the specificity or otherwise of Feulgen staining for DNA appears to have been satisfactorily resolved, if the precautions mentioned earlier are observed, and the remaining major uncertainty is whether the stoichiometry of the reaction is sufficiently reproducible. With the use of a suitable standard sample in all experiments, and with the measurements confined to relative values, this objection also appears to have been met, so that microspectrophotometry after Feulgen staining emerges as the most practical of the three cytophotometric

methods. A quartz microscope is not required, satisfactory optical conditions in the specimen can readily be achieved by the choice of a mounting medium of suitable refractive index, and elaborate ancillary apparatus is not necessary, particularly if photographic photometry is to be used.

For all these reasons, therefore, photometry after Feulgen staining was the preferred technique for the subsequent experiments in the series. The large number of laboratories, mainly in the United States, reporting results obtained by Feulgen photometry, and the very few making use of either ultraviolet microspectrophotometry or the methyl green technique, implies that the majority of other workers in this field have reached similar conclusions.

4.3. The relative deoxyribonucleic acid contents of nuclei from various rat tissues.

In the experiments here reported, the amounts of DNA contained in individual nuclei from seven rat tissues have been compared, the tissues being normal kidney, liver, intestine, pancreas and spermatozoa, regenerating liver following partial hepatectomy, and liver bearing tumours induced by administration of dimethylaminoazobenzene. Within each tissue, the values for single nuclei show a considerable scatter, but when the data are collected in the form of frequency histograms, it is clearly seen that certain quantities of DNA are found more frequently than

others. In normal kidney, the scatter of values is fairly small, and the mean of the group is approximately twice the mean value found for rat spermatozoa. In liver, on the other hand, the nuclei do not fall into a single group, but rather into two main groups, one having a mean DNA value very close to the mean value for kidney nuclei, and the second group having approximately double the mean value. These two groups have been designated Class I and Class II respectively. In addition, normal liver contains a small proportion of nuclei whose mean DNA content is about twice that of the Class II nuclei, i.e., eight times that of the sperm head, these nuclei being regarded as belonging to Class III.

The majority of the nuclei in the somatic tissues studied were found to belong to Class I, while Class II nuclei were found in considerable numbers in normal liver, tumour bearing liver, regenerating liver, and in pancreas. A very few Class II nuclei were detected in normal kidney and in intestine. Class III nuclei, though infrequent, are present in normal liver, and in increased proportion in liver regenerating after hepatectomy and in tumour-bearing liver. Class IV nuclei, having about 16 times the amount of DNA contained in the sperm head, occur somewhat rarely in regenerating liver and in tumour bearing liver, but are apparently absent from normal liver (or at least occur so

infrequently that none were found in the samples measured).

Within each of these major classes of nuclei, there is a considerable scatter of values, and particularly in the classes having the higher DNA contents, although each class tends to form a symmetrical peak in the frequency histogram. Statistical assessment of the results suggests that this scatter of values is greater than can be accounted for by the errors of measurement. Thus there is a true variation between the nuclei in each group, the coefficient of variation being of the order of 5-15 per cent in the case of liver and kidney. The extent of the variation has not been worked out for all the tissues studied, the data collected being insufficient for full statistical assessment, but inspection of the frequency histograms suggests that the situation is similar in other tissues also.

Chemical analysis and counting of nuclei in bulk have shown that in adult, non-dividing tissues the mean amount of DNA per nucleus is fairly constant, the somatic tissues, in general, having nuclei whose DNA content is about twice the value found in the mature sperm head (Mirsky and Ris, 1951; Vendrely and Vendrely, 1948, 1949; Thomson, Heagy, Hutchison and Davidson, 1953). Some workers, notably Boivin, Vendrely and Vendrely (1948) have suggested that in any species all the somatic nuclei contain the same amount of DNA, although the chemical estimation on which this view

was founded can only yield a mean value for the large number of nuclei measured. One serious objection to what one may call the Boivin-Vendrely hypothesis is the observation that in rat liver the mean DNA value per nucleus is considerably higher than that found in other rat tissues (Harrison, 1951; Leuchtenberger, Vendrely and Vendrely, 1951; Thomson et al., 1953). However, purely histological observations have demonstrated, in rat liver, the presence of a proportion of polyploid cells, i.e., cells carrying more than the diploid chromosome number, and have also shown that the proportion of such cells increases with increasing age of the animal (Beams and King, 1942; McKellar, 1949; Sulkin, 1943). If there is in fact some relationship between the number of sets of chromosomes in a nucleus and the amount of DNA which it contains, then one may combine the histological observations with the chemical ones to give a modified hypothesis, namely that the amount of DNA contained within one haploid set of chromosomes in a single species is constant, rather than the amount of DNA contained within one nucleus. If this modified hypothesis is correct, then one would expect the majority of somatic tissue nuclei to have twice the amount of DNA contained in the gametes, while those nuclei which are tetraploid would have four times the haploid amount. As regards normal rat liver and kidney, the results obtained in the present experiments are in remarkably good agreement

with the modified hypothesis outlined above, if we take the Class I, II and III nuclei in liver as representing diploid, tetraploid and octoploid chromosome contents respectively. Also, the proportions of Class I, II and III nuclei found correspond reasonably well with the proportions found by Blesele (1944) for diploid, tetraploid and octoploid nuclei in rat liver.

It is, however, necessary to consider the implications of the hypothesis when applied to tissues other than rat liver and kidney, and particularly in the case of pancreas. Thomson, Heagy, Hutchison and Davidson (1953) have reported mean DNA values for rat pancreas nuclei which are only slightly higher than the mean values which they found for rat kidney nuclei, whereas the results found by cytophotometry suggest that some 19% of pancreas nuclei may come into the tetraploid category. If this is so, then the ratio $\frac{\text{Mean DNA per pancreas nucleus}}{\text{Mean DNA per kidney nucleus}}$ should be about 1.23/1. Jacobj (1925) has suggested, on the basis of measurements of nuclear volume, that about 20% of the nuclei in rat pancreas are tetraploid, a figure in good agreement with the results found by cytophotometry. Possibly the low ratio found by Thomson et al. may be due to selective loss of tetraploid nuclei during isolation.

Not all workers have been in agreement with the modified Boivin-Vendrely hypothesis. Pasteels and Lison

(1950), for example, have carried out cytophotometric investigations on a range of tissues from one rat only, and have concluded that in rat liver and pancreas the nuclei fall into three classes with respect to their DNA contents, the ratios of the means of the three groups being 1:2:4. However, they also report that the DNA content of each class in these two tissues is about 30% lower than the corresponding mean value for the same classes in other tissues from the same animal, and they therefore disagree with the idea of a constant amount of DNA per chromosome set. More recently, however, Pasteels and Lison (1953) have reinvestigated the matter, firstly by repeating (and confirming) their cytophotometric measurements on the same specimens as those which they studied earlier, and secondly, by carrying out similar experiments on liver nuclei from a further three rats. In their second group of experiments, however, they find, in agreement with the results here reported, that the average DNA content of Class II liver nuclei corresponds to the tetraploid value, as estimated from measurements on nuclei from other tissues. They still claim, however, that the findings in their first experiment are valid, and insist that the discrepancy between the two sets of results is evidence against the concept of a constant amount of DNA per set of chromosomes. No confirmation for their earlier findings, however, has come from any other laboratory, nor have

Pasteels and Lison been able to confirm them from another rat, and it seems not improbable that their discordant findings arose from some experimental artefact, possibly in the Feulgen staining process.

Dissenting views on the relationship between DNA content and chromosome numbers have also been expressed by Naora (1951) whose estimations on rat liver nuclei suggest that the ratios of the DNA contents of the three classes of nuclei are 9.5; 13.5; 17.5 instead of 1:2:4. However, the number of nuclei measured by Naora is very small (four of Class II and two of Class III), so that the mean values for these groups cannot have much significance. More recent work by Naora and co-workers is in agreement with the present findings as regards kidney, pancreas and liver, though values lower than the diploid DNA content were found in oesophageal mucous membrane cells (Sibatani, Fukuda, Matsuda and Naora, 1952). This latter discrepancy may be related to the degenerative processes occurring in the cells of a stratified squamous epithelium, the nuclei becoming progressively smaller and less basophilic as they approach the free surface prior to desquamation. The degenerative changes of pyknosis are associated with loss of DNA from the nucleus (Leuchtenberger, 1950; Korson, 1951).

If there is a direct relationship between the chromosome content of a nucleus, and its quota of DNA, then

one would expect some evidence of an increase in DNA content to occur in a nucleus preparing for mitosis. Had the peaks in the frequency histograms of the DNA contents of nuclei from normal rat tissues been non-symmetrical, with a predominance of nuclei showing values higher than the diploid or tetraploid amount of DNA, it would have been tempting to ascribe such values to the presence in resting tissue of a number of nuclei preparing for mitosis. The proportion of nuclei actually in mitosis in adult non-dividing tissues is of course extremely small, and therefore nuclei with DNA contents approaching the theoretical tetraploid value (in preparation for mitosis) would be scanty. However, some information on this aspect was sought by making measurements on nuclei from tissues in which cell growth and multiplication was considerably more rapid than normal, namely liver regenerating after hepatectomy, and chemically induced liver tumours.

In rat liver following partial hepatectomy, there is a period of extremely rapid cell multiplication, so that the number of cells may double within five days. During this period, the mean DNA content per nucleus, as shown by chemical estimations, increases by about half (Thomson, Heagy, Hutchison and Davidson, 1953). The cytophotometric measurements, however, show a reduction in the proportion of Class II cells, the appearance of a considerably increased

proportion of Class III cells, and a few Class IV cells are also present. In this case there is some reason to doubt whether the cells belonging to Classes III and IV do correspond to octoploid and sexbuploid cells, or whether they are in fact tetraploid and octoploid cells which have almost doubled their content of DNA, in preparation for mitosis. Having regard to the very high mitotic rate found in this tissue, it seems likely that the second explanation is the correct one, i.e., that the apparently large proportion of Class III nuclei is not from interphase octoploid cells, but is derived from premitotic tetraploid cells just prior to division into two daughter tetraploid cells. Any such class of cells would only be numerous in a very rapidly proliferating tissue, with a high mitotic index, and even in tumour growth the proportion would be quite small. In tissues where cell division is practically absent, it is likely that any appreciable number of Class III cells present represents a true octoploid group, unless there is evidence for the reduplication of DNA a considerable time in advance of the onset of visible mitosis.

The actual sequence of events as regards DNA duplication and cell division has been studied by a number of workers using a variety of techniques. Thus Price and Laird (1950) have shown that in rat liver following partial hepatectomy, there is an increase in the total amount of

DNA in the residual liver cell nuclei, the amount increasing before the onset of the wave of mitosis. Howard and Pelc (1951) have studied, by autoradiography, the incorporation of radioactive phosphorus into the nuclei of dividing bean root tip cells, and find that the uptake of isotope occurs during interphase. Swift (1950a,b) finds that prophase nuclei have a higher DNA content than interphase nuclei, but Pasteels and Lison (1950) claim that the new accumulation of DNA occurs after, not before, mitosis. However, their conclusions are based on comparisons of measurements on Feulgen stained nuclei at metaphase and telophase, where the optical conditions within the nucleus are vastly different, and their measurements on metaphase chromosomes must be regarded, on optical grounds, as suspect. Walker and Yates (1952b), using ultraviolet absorption measurements on chick fibroblast nuclei in tissue culture, as well as photometric measurements following Feulgen staining, have shown that there is a doubling of the amount of ultraviolet-absorbing material present, during the period from telophase through interphase to just before prophase, while their Feulgen measurements confirm that DNA synthesis occurs during interphase. They consider that part of the increased amount of ultraviolet-absorbing material may represent ultraviolet-absorbing precursors of the DNA molecule. The comparatively small number of measurements, in the present

series, on nuclei from chick heart fibroblast cultures show that while a majority of the cells measured had approximately the same DNA content, a number had DNA values greater than these presumably diploid cells, but less than the theoretical tetraploid value. These findings are certainly in accord with the concept of the amount of DNA increasing during the period prior to prophase, though of course the lack of information on the stage of each nucleus relative to the mitotic cycle makes such views largely speculative. The time-lapse cinematography technique used by Walker and Yates (1952b) is a valuable contribution in this respect.

4.4. General Discussion. The role of deoxyribonucleic acid in the life of the cell.

Deoxyribonucleic acid is a ubiquitous constituent of living cells, occurring in plants, animals and microorganisms, both bacteria and fungi. It is also found in certain of the viruses, those which do not contain it having ribonucleic acid in lieu. The precise role of DNA in the general processes of life is not yet fully clear, but its universal distribution in living things is alone sufficient evidence that its functions must be fundamental to the life of the cell. There is convincing evidence, both from histochemical and macrochemical procedures, that DNA is confined to the cell nucleus, and its association

with the chromosomes (demonstrable both by Feulgen staining and by ultraviolet microscopy during the mitotic process) strongly suggests that it plays some part in the mechanisms of heredity for which the nucleus is largely responsible. This view has received striking support from demonstration of the nature of the transforming factors in microorganisms.

Work on the transforming factors stems from the observations of Griffith (1928) on pneumococci. He showed that a rough (R) non-virulent strain of pneumococci, when injected into mice along with a killed smooth (S) virulent strain of the same organism, gave rise to virulent S organisms of the same capsule type as the killed strain. If however, the living R strain was inoculated along with killed S pneumococci of a different capsule type, the S-type living organisms recovered belonged to the same capsule type as the killed S strain. It appeared likely that the killed organisms, in addition to conferring virulence for the mouse, could also transfer their characteristic capsule type to a non-capsulated strain. In 1931, Dawson and Sia carried out similar transformations in vitro, and showed that the altered strain continued to breed true through many generations and subcultures, thus implying that its genetic characters had been permanently altered. Alloway (1932, 1933) carried this work a stage further, and showed that transformations did not require the whole of the donor cells to be present - transformation of the capsule type

could be achieved through the agency of cell-free extracts from the cells of the donor strain. Some years elapsed before the chemical nature of the active principle was made clear, but in 1944 Avery, MacLeod and McCarty purified the active material responsible for one capsule transformation, and showed that it consisted of deoxyribonucleic acid, free from detectable protein, lipids, or polysaccharides. Further convincing evidence that DNA is in fact the material active in the transformation process was obtained by studies of the effectiveness of the transforming factor after it had been exposed to a number of enzyme preparations. Proteolytic enzymes had no effect on its activity, while bacterial enzymes capable of attacking capsular polysaccharides also left the factor fully active. In 1946, McCarty and Avery showed that minute amounts of highly purified pancreatic deoxyribonuclease rapidly and completely inactivated the transforming factor. The amounts of the purified principle necessary to effect transformation are very small, about 10^{-6} µg of DNA being the amount required to transform one pneumococcus cell (Stocker, 1954).

It thus appears to be fully established that DNA is capable of profoundly modifying the genetic characters of a proportion of bacterial cells with which it comes in contact, but it should be noted that the effect is quite specific for a particular genus of organism. DNA derived from other

sources, e.g., higher animals, is totally devoid of bacterial transforming activity, as indeed is DNA derived from another bacterial genus. The new character conferred on the recipient strain is invariably one present in the donor strain, and it is also possible to confer easily detected characters other than that of specific capsule polysaccharide, e.g., somatic protein antigens. It is, of course, highly likely that a preparation of bacterial DNA is capable of conferring several characters on the cells of the recipient strain, but it is only those which are readily detected that can be recognised to have been transferred. Not only does the transforming factor confer a new character on the recipient strain, but it also confers the ability to reduplicate itself. Transforming reactions of a similar character have been demonstrated for a number of microorganisms other than the pneumococci, and in all of them, the characteristics are similar. The active principle in each case has all the properties of a highly polymerised deoxyribonucleic acid, is inactivated by preparations of deoxyribonuclease, and is capable of transferring some genetic character from one strain of organism to another strain within the same genus, which lacks that particular character. (It is interesting to note that the character conferred may be either some particular ability, e.g., the ability to produce a specific capsular polysaccharide, or a disability, such as inability to ferment

a particular sugar. Thus there appears to be a mechanism for displacing or inhibiting an existing active genetic character, in addition to the more obvious effect of making available a new genetic character (Stocker, 1954)).

These observations on microorganisms are obviously of the highest importance, and one deduction to be made from them is that "deoxyribonucleic acid" even from one bacterial source does not represent a single substance. If one particular sample of DNA is capable of acting as a transforming factor with respect to one character only, then there must be at least as many different deoxyribonucleic acids as there are factors capable of carrying out transformations. It seems highly probable that the reactions we observe are only a small proportion of those which might be detected if means for their recognition existed. From the observations so far made on bacterial DNA, it is logical to deduce that the bacterial cell contains a number of specific types of DNA molecule, each representing one or more of its hereditary characters, and transmissible, after reduplication, to subsequent generations of cells. The laboratory observation that some of these macromolecules can be isolated from killed cells, and used to modify the characters of other cells, is really an experimental artefact, but one of the greatest value in giving an insight into the normal role of DNA in the intact cell.

This idea, that DNA molecules may represent the storehouses of chemical information for subsequent generations of cells, as well as for the cell in which they occur, is attractive as a chemical representation of the concept of genes, but it should not be too readily assumed that the problem of the chemical basis of heredity is solved. For example, other nuclear constituents, in particular the nuclear proteins, might be responsible for transmission of the primary synthetic abilities of the cell, the role of DNA being to direct or inhibit these abilities in particular directions. This admittedly vague hypothesis receives some support from the failure to induce transformations in micro-organisms outwith the particular group of organisms from which the factor was obtained, suggesting that the specific molecules of DNA require to interact with some other constituent of cells within the genus being studied, before their action can become apparent. If the specific molecule or molecules of DNA, together with a source of energy and a supply of the necessary amino acids were all that is required for the initiation of the synthesis of, say, an enzyme protein, then DNA derived from a Pneumococcus might be expected to be effective in inducing synthesis of the enzyme in, for example, a Haemophilus in which it is not normally present. (An obvious alternative hypothesis is that DNA from donor Pneumococci cannot penetrate

Haemophilus cells, while DNA from another Haemophilus strain can do so, thereby maintaining the genus specificity of the process.)

Further insight into the role of DNA as a stimulus towards synthesis in particular directions comes from studies on the infection of bacterial cells by specific bacteriophages. The phage particules which attack coliform bacilli contain DNA and protein, and Hershey and Chase (1952) have studied the fate of the two components of the phage particle when a susceptible host cell is attacked. Using phage particles labelled with radioactive phosphorus in the DNA, and radioactive sulphur in the protein, they have shown that the role of the protein is apparently confined to successful attachment of the particle to, and penetration of, the bacterial cell. The protein does not itself enter the host. The phage DNA, on the other hand, enters the bacterial cell, and there acts as a stimulus to synthesis of new phage DNA and new phage protein which are ultimately liberated as the next generation of phage particles. Whether the phage DNA can itself cause de novo synthesis of both virus protein and virus DNA, given the necessary environmental conditions as regards precursor molecules, energy sources and the like, or whether it acts only by diverting existing bacterial DNA and protein synthesis along abnormal lines, we are unable to say with

certainty - what has been established is that in certain microorganisms and viruses, DNA acts specifically to induce synthetic processes in recipient cells, of a pattern of which the recipient cell did not previously appear to be capable.

Turning our attention to the deoxyribonucleic acids of the cells of higher animals, we find that there is, as yet, no direct evidence of DNA functions comparable to that obtained in microorganisms; considerable indirect evidence, however, is available which leads to the view that in higher animals DNA is also intimately related to the transmission of the hereditary characters from one generation of cells to the next. The rate of renewal of DNA has been studied in a number of tissues differing considerably in their rates of production of new cells, and there appears to be a close correlation between the activity of the tissue in this respect and the turnover rate as measured by the incorporation of tracer isotopes into the DNA molecules. Hevesy and Ottesen (1943), for example, found rapid turnover of DNA in intestinal mucosa, spleen and testis, but very slow turnover in normal liver, kidney and brain. Similarly, labelled adenine is but slowly incorporated into the DNA of non-dividing tissues (Furst, Roll and Brown, 1950), but is rapidly incorporated into the DNA of a tissue such as regenerating liver. By contrast, the uptake of labelled

precursor molecules into RNA is not only more rapid than into DNA in the majority of tissues (Davidson and Raymond, 1947), but nuclear RNA appears to be metabolically more active in this respect than cytoplasmic RNA (McIndoe and Davidson, 1952). The incorporation of labelled precursors into DNA is depressed by X-irradiation, which of course lowers the rate of mitosis (Hevesy, 1949). Taken as a whole, this evidence is consistent with the view that in a non-dividing tissue, the DNA in the nuclei is more or less inert as regards dynamic catabolism and resynthesis. With the arrival of a stimulus to mitosis, there is increased synthesis of DNA molecules, presumably in those cells about to undergo mitosis. If mitosis is slowed or stopped, then the synthesis of new DNA molecules slows also, the change being reflected in a fall in the turnover rate for DNA as measured by isotope incorporation.

Unfortunately, this picture is certainly an over-simplified one, since the apparently inert behaviour of DNA from non-dividing cells is demonstable only when adenine or inorganic phosphate is used as the labelled precursor, whereas isotopic carbon is readily incorporated into the DNA purines in non-dividing liver cells if the precursor is formate, glycine (the non-carboxylic carbon atom), or the β-carbon atom of serine (Lepage and Heidelberger, 1951; Furst and Brown, 1951; Elvin and Sprinson, 1950). These

divergent findings may imply the existence of two separate pathways for DNA synthesis, one of which comes into full operation only when very rapid synthesis is required prior to mitosis.

In studies on the rate of incorporation of radioactive phosphorus into DNA, Stevens, Daoust and Leblond (1953) have attempted to correlate the extent of isotope incorporation into the DNA of an organ, with the rate of production of new cell nuclei, and have concluded that if the extent of incorporation of phosphate is a measure of the number of new molecules of DNA produced, then only half of the new DNA is required for the new nuclei. It is difficult to assess the significance of this finding, involving as it does measurements of cell number and highly critical measurements of specific activity, but it may be that even as regards phosphorus incorporation, the DNA of the non-dividing nucleus is not inactive. An interesting hypothesis, advocated by Stevens et al., is that when DNA is being synthesised for the two daughter nuclei, the original DNA of the parent nucleus is not re-used, at least as far as the phosphorus is concerned. Further studies in this direction are required.

If DNA is to be accepted as a probable, or even a possible chemical basis for the concept of the gene, it is obviously necessary to establish that it shows sufficient

chemical heterogeneity to be able to form the thousands of distinct genes known to exist. There are obviously a very large number of possible combinations of the four main nucleotides, if arranged in the form of polynucleotide chains and in equimolecular proportions. It is also clear that variations in the proportions of the nucleotides will permit of a greatly increased number of possible combinations, while the introduction of additional bases (5-methylcytosine, for example) allows for further combinations still. There is thus ample scope, from a chemical point of view, for the existence of an exceedingly large number of distinct deoxyribonucleic acids, although stereochemical considerations may limit the actual number. If the DNA isolated from a single tissue consists of a large number of different molecular species, representing the gene complement of that tissue, then the comparative constancy of the ratios of the bases in that DNA sample may merely reflect a similar degree of constancy in the proportions of the different molecular species present. When we also take into consideration the macromolecular nature of DNA, we see that differences between adjacent DNA molecules need not be confined to nucleotide sequences in the chain, but may involve also the nature of the binding links between adjacent chains, folding or similar structural differences, as well as the positions of breaks, reduplicated regions and other variations in molecular detail.

Experimental evidence for chemical heterogeneity in DNA from a single tissue has been obtained in recent years, particularly through differences in the affinity of the DNA molecules for macromolecules with basic properties, such as the basic proteins globin and histone, or synthetic ion-exchangers such as diethylaminoethylcellulose, such differences having been made the basis for chromatographic separations of purified preparations of DNA into a number of fractions. The resulting fractions of DNA have been shown to differ in the ratios of the bases which they contain, the base ratios being related to the affinities of the DNA molecules for the basic proteins (Chargaff, Crampton and Lipshitz, 1953). Similarly, heterogeneity with respect to transforming ability has been shown for DNA of bacterial origin (Brown and Watson, 1954), although attempts to demonstrate differences in isotope turnover in fractions separated by salt solubility methods have been somewhat inconclusive (Bendich, Russell and Brown, 1953).

Although studies of the heterogeneous nature of DNA are still at an early stage, it is clear that it is misleading to talk of DNA as if it were a single substance, except as a generic term comparable to the use of "protein" or "fat". The time is still far distant when samples of DNA can be prepared, consisting of one molecular species only, unless biological methods can be developed for the

detection of heterogeneity, comparable to, say, the most specific of antigen-antibody interactions. When freed from protein, DNA is not antigenic, and at present virtually the only technique which is valid for the measurement of the biological activity of DNA is the laborious one of detection of transforming activity for microorganisms. This method, as we have seen, is at best only semi-quantitative, and is valid for a strictly limited range of bacterial DNA preparations. No comparable technique yet exists for assessment of DNA from higher animals or plants.

Although the evidence so far presented all points to DNA as the most probable group of substances to form the chemical basis of gene action, it is not justifiable to assume that this is the only possible role for DNA in the economy of the cell. The demonstration of very large amounts of deoxyribosides in the eggs of the sea urchin and the frog (Zeuthen, 1951; Hoff-Jorgensen and Zeuthen, 1952) suggests a function other than that of transmission of hereditary characters only, although, as has been indicated in an earlier section, deoxyribosides are by no means synonymous with highly polymerised DNA.

Other information on roles of DNA besides that of transmission of hereditary factors is scanty. Fraenkel-Conrat and Ducay (1951) have reported that the biotin-binding component of egg-white consists of a complex of

DNA with a protein, while the cytoplasmic kappa-factor which acts as a killer-substance for Paramecium also appears to be a DNA-protein complex (Preer, 1948). Its mode of action, however, does not altogether correspond to the action of a bacteriophage particle, and its role remains obscure. Leuchtenberger and Schrader (1952), using photometric determinations of Feulgen staining, have shown a very great variation (more than thirty times) in the amounts of DNA found in the nuclei of salivary gland cells in a single snail, the amount decreasing with increasing production of the polysaccharide secretion product. They suggest that in this particular case, the role of DNA might well be as a precursor substance rather than as a genetic determinant. (In this experiment, it would seem especially desirable to investigate very closely the specificity of the method used for determination of DNA, and if possible confirm the observations by other methods.)

Thus we can summarise present knowledge of the functions of DNA, as follows:-

1. DNA is invariably present in plant and animal cell nuclei, and is wholly confined to the nucleus, with a few exceptions.
2. In plants and higher animals, increased duplication of nuclear material is accompanied by an increased turnover of DNA as measured by isotope incorporation methods.

3. The amount of DNA contained in a non-dividing nucleus is proportional to the number of chromosome sets which that nucleus contains, and the amount of DNA characteristically associated with one set of chromosomes is constant for a particular species. Within this generalisation, however, small variations from nucleus to nucleus occur.

4. In certain viruses, the DNA component of the infective particle acts as the stimulus, in a host cell, to the production of new virus DNA and protein.

5. In microorganisms, cell extracts, which are capable of producing genetic transformations in other cells within the same genus, consist of highly polymerised DNA.

PART V.

SUMMARY OF EXPERIMENTAL RESULTS.

Summary of Experimental Results.

1. A cytophotometric technique has been developed for the study, by photographic means, of certain constituents of the cell nucleus. The technique is equally applicable in the ultraviolet and visible regions of the spectrum.

2. The photographic cytophotometric technique has been applied to the estimation of the relative amounts of deoxyribonucleic acid contained in individual isolated nuclei from a number of rat tissues, and of embryonic chick cells in tissue cultures.

3. Comparative measurements have been made on the same batches of rat liver and kidney nuclei, using four distinct methods for the estimation of deoxyribonucleic acid in the nuclei, namely estimation of the mean amount of DNA per nucleus by bulk chemical estimation and counting, estimation of the extent of ultraviolet light absorption by single nuclei after treatment with solutions of crystalline ribonuclease, estimation of the intensity of Feulgen staining in single nuclei, and estimation of the intensity of methyl green staining in single nuclei. The reasonably satisfactory agreement between the results by all four methods is considered to be evidence for the validity of the cytophotometric techniques.

4. The nuclei of the tissues studied fall into four classes, as judged by their content of DNA. Using the amount of DNA contained in a sperm head as a unit of comparison, Class I nuclei contain twice that amount of DNA, Class II nuclei four times the amount, Class III nuclei eight times the amount, and Class IV nuclei contain sixteen times the amount of DNA.

5. In rat kidney and intestine, all the nuclei belong to Class I, with only isolated exceptions which belong to Class II. In normal liver and in pancreas, the majority of the nuclei belong to Class I, but about 20% of pancreas nuclei, and 40% of liver nuclei belong to Class II. 1-2% of liver nuclei belong to Class III.

6. A liver which was the site of chemically induced tumour growth showed an increased proportion of Class I nuclei, a correspondingly reduced proportion of Class II nuclei, about 5% of Class III nuclei, and about 1% of Class IV nuclei.

7. A regenerating liver which was examined 48 hours after partial hepatectomy showed about 1% of Class IV nuclei, 15% belonging to Class III, 25% to Class II, and the remainder to Class I.

8. Statistical assessment by analysis of variance suggests that in rat liver and kidney nuclei the variations found within each class can be largely accounted for by the errors of the method, but that there is a true coefficient of variation between nuclei in the same class, of the order of 5-15% or less.

9. The majority of the nuclei present in chick heart fibroblast cultures have DNA contents of the same order as those found in chick embryo liver. In each case, nuclei are found whose DNA content approaches twice that of the majority of the nuclei present.

Bibliography.

Bibliography

Ackermann, D., Hoppe-Seyl. Z., 43, 99 (1904).

Allen, R.J.L., Biochem.J., 34, 858 (1940).

Allfrey, V., Stern, H., Mirsky, A.E. and Saetren, H., J.gen.Physiol., 35, 529 (1952).

Alloway, J.L., J.exp.Med., 55, 91 (1932).

Alloway, J.L., J.exp.Med., 57, 265 (1933).

Altmann, R., "Die Elementarorganismen und ihre Beziehungen sur Zellen." Leipzig, 1890.

Arnesen, K., Goldsmith, Y. and Dulaney, A.D., Cancer Res., 9, 669 (1949).

Astbury, W.T., Symp. Soc. exp. Biol., 1, 66 (1947).

Astbury, W.T. and Bell, F.O., Nature, London, 141, 747 (1938).

Avery, O.T., MacLeod, C.M. and McCarty, M., J.exp.Med., 79, 137 (1944).

Barnard, J.E. and Elford, W.J., Proc.Roy.Soc.London, B 109, 360 (1931).

Barton, J., Biol.Bull., Wood's Hole, 103, 326 (1952).

Beams, H.W. and King, R.L., Anat.Rec., 83, 281 (1942).

Beebe, S.P. and Shaffer, B., Amer.J.Physiol., 14, 231 (1905).

Behrens, M., Hoppe-Seyl.Z., 209, 59 (1932).

Behrens, M., Hoppe-Seyl.Z., 220, 97 (1933).

Behrens, M., Hoppe-Seyl.Z., 232, 263 (1935).

Behrens, M., Hoppe-Seyl.Z., 253, 185 (1938).

Behrens, M., Hoppe-Seyl.Z., 258, 27 (1939).

Bendich, A., Russel, P.J. and Brown, G.B., J.biol.Chem., 203, 305 (1953).

Biesele, J.J., Cancer Res., 4, 232 (1944).

Blumel, J. and Kirby, H., Proc.nat.Acad.Sci., Wash. 34, 561 (1948).

Boivin, A., Vendrely, R. and Vendrely, C., C.R.Acad.Sci., Paris, 226, 1061 (1948).

Bowen, E.J., "Chemical Aspects of Light", Clarendon Press, Oxford (1946).

Brachet, J., C.R.Soc.Biol., Paris, 133, 88 (1940).

Brachet, J., Arch.Biol., 53, 207 (1941).

Brachet, J., Experientia, 2, 142 (1946).

Brandenburg, K., Munch.med.Wschr., 47, 183 (1900).

Brown, G.L. and Watson, M., Nature, London, 172, 339 (1953).

Brown, G.L. and Watson, M., Unpublished results, reported at a Colloquium on Bacterial Genetics, London, December 1954.

Brownlee, K.A., "Industrial Experimentation", H.M. Stationery Office, London (1949).

Brumberg, E.M., C.R.Acad.Sci., U.R.S.S., 25, 473 (1939).

Brumberg, E.M., Nature, London, 152, 357 (1943).

Burch, C.R., Proc.phys.Soc., London, 59, 41 (1947).

Callan, H.G., Nature, London, 152, 503 (1943).

Caspersson, T., Skand.Arch.Physiol., 73, Supp.8,1 (1936).

Caspersson, T., Arch.exp.Zellforsch., 22, 655 (1939).

Caspersson, T., J.Roy.micr.Soc., 60, 8 (1940a).

Caspersson, T., Chromosoma, 1, 605 (1940b).

Caspersson, T., Naturwiss., 29, 33 (1941).

Caspersson, T., Symp.Soc.exp.Biol., 1, 127 (1947).

Caspersson, T., Exp.cell Res., 1, 595 (1950a).

Caspersson, T., "Cell Growth and Cell Function", Norton and Co., New York (1950b).

Caspersson, T., Exp.cell Res., 7, 598 (1954).

Caspersson, T., Experientia, 11, 45 (1955).

Caspersson, T., Jacobsson, F. and Lomakka, G., Exp.cell Res., 2, 301 (1951).

Caspersson, T. and Schultz, J., Nature, London, 142, 294 (1938).

Caspersson, T. and Schultz, J., Proc.nat.Acad.Sci., Wash., 26, 507 (1940).

Caspersson, T. and Schultz, J., in "Genetics in the 20th Century", The MacMillan Company, New York (1951).

Catchpole, H.R. and Gersh, I., Trans.Farad.Soc., 1131, (1950).

Caventou, J.B., Ann.chim.Phys., 31, 337 (1826).

Cecil, R. and Ogston, A.G., J.chem.Soc., 1382, (1948).

Chargaff, E., Experientia, 6, 201 (1950).

Chargaff, E., Fed.Proc., 10, 654 (1951).

Chargaff, E., in "The Nucleic Acids", Vol.1., ed. Chargaff, E., and Davidson, J.N., Academic Press, New York (1955).

Chargaff, E., Crampton, C.F. and Lipshitz, R., Nature, London, 172, 289 (1953).

Chargaff, E. and Lipshitz, R., J.Amer.chem.Soc., 75, 3658 (1953).

Chargaff, E. and Zamenhoff, S., J.biol.Chem., 173, 327 (1948).

Chargaff, E., Zamenhoff, S., Brauerman, G. and Kerin, L., J.Amer.chem.Soc., 72, 3825 (1950).

Claude, A., Science, 97, 451 (1943).

Claude, A., J.exp.Med., 84, 51 (1946).

Cohen, S.E. and Stanley, W.M., J.biol.Chem., 144, 589 (1942).

Cole, P.A. and Brackett, F.S., Rev.sci.Instr., 11, 419 (1940).

Coleman, L.C., Stain Technol., 13, 123 (1938).

Commoner, B., Science, 110, 31 (1949).

Commoner, B., Science, 111, 71 (1950).

Commoner, B. and Lipkin, D., Science, 110, 41 (1949).

Conway, E.E., Gilbert, L. and Butler, J.A.V., J.chem.Soc., 3421, (1950).

Creeth, J.M., Culland, J.M. and Jordan, D.O., J.chem.Soc., 1141, (1947).

Crosbie, G.W., Smellie, R.M.S. and Davidson, J.N., Biochem.J., 54, 237 (1953).

Crossman, G., Science, 85, 250 (1937).

Danielli, J.F., "Cytochemistry", Chapman and Hall, Ltd., London (1953).

Davidson, J.N. and Leslie, I., Exp.cell Res., 2, 366 (1951).

Davidson, J.N. and Raymond, W., Biochem.J., 42, proc. xiv, (1947).

Davidson, J.N. and Smellie, R.M.S., Biochem.J., 52, 599 (1952).

Davidson, J.N. and Waymouth, C., J.Physiol., 105, 191 (1946).

Davidson, W.M. and Smith, D.R., Brit.med.J., 1954(2), 6 (1954).

Dawson, M.H. and Sia, R.H.P., J.exp.Med., 54, 681 (1931).

de Tomasi, J.A., Stain Technol., 11, 137 (1936).

Doty, P. and Bunce, B., J.Amer.chem.Soc., 74, 5029 (1952).

Dounce, A.L., J.biol.Chem., 147, 685 (1943).

Dounce, A.L., Ann.N.Y.Acad.Sci., 50, 982 (1950).

Dounce, A.L. and Beyer, G.T. J.biol.Chem., 175, 159 (1948).

Dounce, A.L. and Lan, T.H., Science, 97, 584 (1943).

Dounce, A.L., Tishkoff, G.H., Barnet, S.R. and Freer, R.M., J.gen.Physiol., 33, 629 (1950).

Edstrom, J.E., Nature, London, 172, 809 (1953).

Ehrlich, P., Verh. physiol.Ges. Berlin, 20 (1878).

Elson, D. and Chargaff, E., Phosphorus Metabolism, 2, 331 (1952).

Elwin, D. and Sprinson, D., J.Amer.chem.Soc., 72, 3317 (1950).

Feulgen, R., Hoppe-Seyl.Z., 135, 203 (1918).

Feulgen, R., "Chemie und Physiologie der Nucleinstoffe" Borntraeger, Berlin (1923).

Feulgen, R. and Rossenbeck, H., Hoppe-Seyl.Z., 135, 203 (1924).

Fischer, A., "Fixierung, Farbung und Bau des Protoplasmas" Leipzig, (1899).

Fletcher, W.E., Thesis, University of London, 1948 "On the Structure of the Nucleic Acids".

Fraenkel-Conrat, H. and Ducay, E.D., Biochem.J., 49, proc. xxxix (1951).

Fraenkel-Conrat, H., Snell, N.S. and Ducay, E.D., Arch.Biochem.Biophys., 39, 80 (1952).

Furberg, S., Acta chem.Scand., 6, 634 (1952).

Furst, S.S. and Brown, G.B., J.biol.Chem., 191, 239 (1951).

Furst, S.S., Roll, P.M. and Brown, G.B., J.biol.Chem., 183, 251 (1950).

Gersh, I., Anat.Rec., 53, 309 (1932).

Glick, D., "Techniques of Histochemistry and Cytochemistry" Interscience Publishers, New York (1949).

Greenstein, J.P., Adv. in Protein Chem., 1, 210 (1944).

Griffin, A.C., Nye, W.N., Noda, L. and Luck, J.M., J.biol.Chem., 176, 1225 (1948).

Griffith, F., J.Hyg., 27, 113 (1928).

Gulick, A., Adv. in Enzymol., 4, 1 (1946).

Gulland, J.M., Barker, G.R. and Jordan, D.O., Ann.Revs.Biochem., 14, 175 (1945).

Gulland, J.M., Jordan, D.O., and Taylor, H.F.W., J.chem.Soc., 1131, (1947).

Gulland, J.M., Jordan, D.O. and Threlfall, C.J., J.chem.Soc., 1129, (1947).

Hardy, W.B., J.Physiol., 24, 158 (1899).

Harrison, M.F., Nature, London, 168, 248 (1951).

Henle, W., Henle, G. and Chambers, L.A., J.exp.Med., 68, 335 (1938).

Hershey, A.D. and Chase, M., J.gen.Physiol., 36, 39 (1952).

Hevesy, G., Nature, London, 163, 869 (1949).

Hevesy, G. and Ottesen, J., Acta physiol.Scand., 5, 237 (1943).

Higgins, G.M. and Anderson, R.M., Arch.Path., 12, 186 (1931).

Hillary, B.B., Botan.Gaz., 101, 276 (1939).

Hoff-Jorgensen, E., in "Recent Developments in Cell Physiology" ed. Kitching, J.A., Butterworths, London (1954).

Hoff-Jorgensen, E. and Zeuthen, E., Nature, London, 169, 245 (1952).

Hogeboom, G.H., Schneider, W.C. and Pallade, G., J.biol.Chem., 172, 619 (1948).

Hoppe-Seyler, F., Med.chem.Untersuch., Berlin, 4, 441 (1871).

Hotchkiss, R.D., J.biol.Chem., 175, 315 (1948).

Howard, A. and Pelc, S.R., Exp.cell Res., 2, 178 (1951).

Irwin, J.L. and Irwin, E.M., Science, 110, 426 (1949).

Irwin, J.L. and Irwin, E.M., Fed.Proc., 11, 255 (1952).

Jacobj, W., Arch.Entw-mech.Org., 106, 124 (1925).

Jeener, R., Experientia, 2, 458 (1946).

Jeener, R., Actualités Biochim., 10, 88 (1947).

Johnson, B.K., Proc.phys.Soc., London, 42, 16 (1929).

Jones, W., "Nucleic Acids", Longmans Green and Co., London (1914).

Jones, W., Amer.J.Physiol., 52, 195 (1920).

Jorpes, E., Biochem.Zeitschr., 151, 227 (1924).

Jorpes, E., Acta med.Scand., 68, 253 (1928).

Jungner, I., Acta physiol.Scand., 20, Supp.69, 1 (1950).

Kasten, F.H., J.Histochem.Cytochem., 3, 387 (1955).

Katz, S., J.Amer.chem.Soc., 74, 2238 (1952).

Kay, E.R.M. and Dounce, A.L., J.Amer.chem.Soc., 75, 4041 (1953).

Klebs, E., Z.med.Wiss., 6, 417 (1868).

Kohler, A., Z.fur wissen.Mikr., 21, 129 (1904).

Korson, R., J.exp.Med., 93, 121 (1951).

Kossel, A., Hoppe-Seyl.Z., 5, 152 (1881).

Kossel, A., Hoppe-Seyl.Z., 7, 7 (1882).

Kossel, A., Hoppe-Seyl.Z., 8, 511 (1884).

Kossel, A., Hoppe-Seyl.Z., 22, 188 (1896).

Kossel, A., "The Protamines and Histones", Longmans Green and Co., London (1928).

Kossel, A. and Steudel, H., Hoppe-Seyl.Z., 37, 245 (1902).

Kossel, A. and Steudel, H., Hoppe-Seyl.Z., 38, 49 (1903).

Kunitz, M., J.gen.Physiol., 24, 15 (1940).

Kurnick, N.B., J.gen.Physiol., 33, 243 (1950a).

Kurnick, N.B., Exp.cell.Res., 1, 151 (1950b).

Kurnick, N.B., Exp.cell.Res., 3, 649 (1952).

Kurnick, N.B. and Foster, M., J.gen.Physiol., 34, 147 (1950).

Kurnick, N.B. and Herskowitz, I.H., J.cell.comp.Physiol., 39, 281 (1952).

Kurnick, N.B. and Mirsky, A.E., J.gen.Physiol., 33, 265 (1950)

Lavin, G.I., Rev.sci.Instr., 14, 375 (1943).

Lee, P. and Gray, D., unpublished work quoted by Scott, J.F. and Sinsheimer, R.L., in "Medical Physics", Vol.II. Year Book Publishers, Inc., Chicago (1950).

Leitgeb, H., Mitt.bot.Inst., Graz (1888).

Lepage, G.A. and Heidelberger, C., J.biol.Chem., 188, 593 (1951).

Lessler, M.A., Arch.Biochem., 32, 42 (1951).

Leuchtenberger, C., Chromosoma, 3, 449 (1950).

Leuchtenberger, C., Klein, G. and Klein, E., Cancer Res., 12, 480 (1952).

Leuchtenberger, C., Leuchtenberger, R., Vendrely, C. and Vendrely, R., Exp.cell.Res., 3, 240 (1952).

Leuchtenberger, C. and Schrader, F., Proc.nat.Acad.Sci., Wash., 38, 99 (1952).

Leuchtenberger, C., Vendrely, R. and Vendrely, C., Proc.nat.Acad.Sci., Wash., 37, 33 (1951).

Levene, P.A. and Bass, L.W., "Nucleic Acids". Chemical Catalogue Co., New York. (1931).

Levene, P.A. and Jacobs, W.A., Ber.d.d.Chem.Ges., 42, 3247 (1909).

Levene, P.A. and Jacobs, W.A., J.biol.Chem., 12, 411 (1912).

Levene, P.A. and London, E.S., J.biol.Chem., 83, 793 (1929).

Lhotka, J.F. and Davenport, H.A., Stain Technol., 26, 35(1951)

Lillienfeld, L., Hoppe-Seyl.Z., 18, 473 (1894).

Lison, L., "Histochimie Animale", Gauthier-Villars, Paris (1936).

Lucas, F.F. and Stark, M.B., J.Morphol.Physiol., 52, 91 (1931).

McCarty, M. and Avery, O.T., J.exp.Med., 83, 89 (1946).

McDonald, M.R., J.gen.Physiol., 32, 59 (1948).

McIndoe, W.M. and Davidson, J.N., Brit.J.Cancer, 6, 200 (1952).

McKellar, M., Amer.J.Anat., 85, 363 (1949).

Magasanik, B., in "The Nucleic Acids", Vol.I, Ed.Chargaff,E. and Davidson, J.N., Academic Press, New York. (1955).

Mann, G., "Physiological Histology", London (1902).

Manton, F. and Smiles, J., Ann.Bot., 7, 195 (1943).

Markham, R. and Smith, J.D., Biochem.J., 45, 294 (1949).

Marshak, A., J.gen.Physiol., 25, 275 (1941).

Mayer, D.T. and Gulick, A., J.biol.Chem., 146, 433 (1942).

Miescher, F., Med.-chem.Untersuch., 4, 441 (1871).

Miescher, F., Verh.naturf.Ges., Basle, 6, 138 (1873).

Miescher, F., Ber.d.d.chem.Ges., 7, 376 (1874).

Miescher, F., "Histochemischen und Physiologischen Arbeiten", Vogel, Leipzig (1897).

Millon, A.N.E., C.R.Acad.Sci., Paris 18, 1041 (1844).

Mirsky, A.E., Adv. in Enzymol., 3, 1 (1943).

Mirsky, A.E. and Pollister, A.W., J.gen.Physiol., 30, 117 (1946).

Mirsky, A.E. and Ris, H., J.gen.Physiol., 31, 1 and 7 (1947).

Mirsky, A.E. and Ris, H., J.gen.Physiol., 34, 475 (1951).

Molovodov, P.F., Protoplasma, 25, 570 (1936).

Moore, B.C., Chromosoma, 4, 563 (1952).

Moore, K.L., Graham, M.A. and Barr, M.L., Surg.Gynaecol. Obstet., 96, 641 (1953).

Myrback, K. and Jorpes, E., Hoppe-Seyl.Z., 237, 159 (1935).

Naora, H., Science, 114, 279 (1951).

Naora, H., Science, 115, 248 (1952).

Neumann, A., Archiv.f.Anat.u.Physiol., p.374 (1898).

Novikoff, A.B., Hecht, L., Podber, E. and Ryan, J., J.biol.Chem., 194, 153 (1952).

Novikoff, A.B., Podber, E. and Ryan, J., Fed.Proc., 9, 210 (1950).

Ogur, M. and Rosen, G.I., Arch.Biochem., 25, 262 (1950).

Opie, E.L. and Lavin, G.I., J.exp.Med., 84, 107 (1946).

Ornstein, L. and Pollister, A.W., Science, 116, 203 (1952).

Overend, W.G. and Stacey, M., Nature, London, 163, 538 (1949).

Pasteels, J. and Lison, L., Arch.Biol., Paris, 61, 445 (1950a).

Pasteels, J. and Lison, L., C.R.Acad.Sci., Paris, 230, 780 (1950b).

Pasteels, J. and Lison, L., C.R.Acad.Sci., Paris, 236, 236 (1953).

Pauling, L.K. and Corey, R.B., Nature, London, 171, 346 (1953).

Pearse, A.G.E., "Histochemistry, Theoretical and Applied", Churchill, London (1953).

Petermann, M.L., Slater, R.B. and Larack, A.M., Cancer, 2, 510 (1949).

Piccard, J., Ber.d.d.Chem.Ges., 7, 1714 (1874).

Pollister, A.W., Rev.Hémat., 5, 527 (1950).

Pollister, A.W., Exp.cell.Res., 1952 Supp.2, 59 (1952).

Pollister, A.W., Lab.Investigation 1, 106 (1952a).

Pollister, A.W., Lab.Investigation, 1, 231 (1952b).

Pollister, A.W. and Leuchtenberger, C., Nature, London, 163, 360 (1949a).

Pollister, A.W. and Leuchtenberger, C., Proc.nat.Acad.Sci., Wash., 35, 66 (1949b).

Pollister, A.W. and Leuchtenberger, C., Proc.nat.Acad.Sci., Wash., 35, 111 (1949c).

Pollister, A.W. and Mirsky, A.E., J.gen.Physiol., 30, 101 (1946).

Pollister, A.W. and Moses, M.J., J.gen.Physiol., 32, 567 (1949).

Pollister, A.W. and Ris, H., Cold Spring Harbor Symp.quant. Biol., 12, 147 (1947).

Pollister, A.W. and Swift, H.H., Science, 111, 68 (1950).

Pollister, A.W., Swift, H.H. and Alfert, M., J.cell.comp. Physiol., 38, Supp.1, 101 (1951).

Preer, J.R., Genetics, 33, 625 (1948).

Price, J.M. and Laird, A.K., Cancer Research, 10, 650 (1950).

Price, J.M., Miller, E.C. and Miller, J.A., J.biol.Chem., 173, 345 (1948).

Raspail, F.V., Ann.Sci.Obs., 1, 72 (1829).

Raspail, F.V., "Essai de Chimie Microscopique Appliquée à la Physiologie", Paris, (1830).

Ris, H. and Mirsky, A.E., J.gen.Physiol., 33, 125 (1949).

Schmidt, G. and Thannhauser, S.J., J.biol.Chem., 161, 83 (1945).

Schneider, W.C. J.biol.Chem., 161, 293 (1945).

Schneider, W.C., J.biol.Chem., 165, 585 (1946).

Schneider, W.C., J.biol.Chem., 176, 259 (1948).

Schneider, W.C., Hogeboom, G.H. and Ross, H.E., J.nat.Cancer Inst., 10, 977 (1950).

Schneider, W.C. and Potter, V.R., J.biol.Chem., 177, 893 (1949).

Scott, J.F. and Sinsheimer, R.L., in "Medical Physics", Vol.ii, Ed.Glasser, O., Year Book Publishers, Inc., Chicago (1950).

Seeds, W.E., in "Progress in Biophysics and Biophysical Chemistry", Vol.iii, Ed. Butler, J.A.V. and Randall, J.T. Pergamon Press, London (1953).

Sibatani, A., Fukuda, M., Matsuda, H. and Naora, H., J.Biochem.Japan, 39, 63 (1952).

Smellie, R.M.S., McIndoe, W.M., Logan, R., Davidson, J.N. and Dawson, I.M., Biochem.J., 54, 280 (1953).

Smith, D.B. and Shaffer, H., Canad.J.Res., B28, 96 (1950).

Snedecor, G.W., "Statistical Methods", Iowa State College Press, Iowa (1946).

Sparrow, A.H. and Hammond, M.R., Amer.J.Bot., 34, 439 (1947).

Stedman, E. and Stedman, E., Nature, London, 152, 267 (1943).

Stedman, E., and Stedman, E., Biochem.J., 38, Proc.xxiv (1944).

Stedman, E. and Stedman, E., Cold Spring Harbor Symp.quant. Biol., 12, 234 (1947).

Stedman, E. and Stedman, E., Biochem.J., 47, 508 (1950).

Stedman, E. and Stedman, E., Phil.Trans.Roy.Soc., (B) 235, 565 (1951).

Stern, H., Mirsky, A.E., Allfrey, V. and Saetren, H., J.gen.Physiol., 35, 559 (1952).

Stern, K.G., Exp.cell Research, Supp.2, p.1 (1952).

Steudel, H., Hoppe-Seyl.Z., 55, 407, and 56, 212 (1908).

Stevens, C.E., Laoust, R., Leblond, C.P., J.biol.Chem., 202, 177 (1953).

Stirling, W., J.Anat.Physiol., Leipzig, 10, 185 (1875).

Stocker, B.A.D., Unpublished results, quoted at a Colloquium on Bacterial Genetics, London, December 1954.

Stoneburg, C.A., J.biol.Chem., 129, 189 (1939).

Stowell, R.E., J.nat.Cancer Inst., 3, 111 (1942).

Swift, H.H., Physiol.Zool., 23, 169 ~~319~~ (1950a).

Swift, H.H., Proc.nat.Acad.Sci., Wash., 36, 643 (1950b).

Swift, H.H., Int.Rev.Cytol., 2, 1 (1953).

Swift, H.H., in "The Nucleic Acids", Vol.II, Ed. Chargaff, E. and Davidson, J.N., Academic Press, New York (1955).

Swingle, W.T. and Briggs, L.J., Science, 26, 180 (1907).

Taft, E.B., Exp.cell.Research, 2, 312 (1951).

Tamm, C., Hodes, M.E. and Chargaff, E., J.biol.Chem., 195, 49 (1953).

Thomson, R.Y., Heagy, F.C., Hutchison, W.C. and Davidson, J.N., Biochem.J., 53, 460 (1953).

Thorell, B., Acta Med.Scand., 129, Supp. CC (1947).

Thorell, B., Trans.Faraday Soc., 1234, (1950).

Uber, F.M., Amer.J.Bot., 26, 799 (1939).

Vendrely, R. and Vendrely, C., Experientia, 4, 434 (1948).

Vendrely, R. and Vendrely, C., Experientia, 5, 327 (1949).

Vercauteren, R., Enzymologia, 14, 134 (1950).

Vischer, E. and Chargaff, E., J.biol.Chem., 176, 703 (1948).

Volkin, E. and Carter, C.E., J.Amer.chem.Soc., 73, 1516 (1951).

Volkin, E. and Cohn, W.E., J.biol.Chem., 203, 319 (1953).

Walker, P.M.B. and Davies, H., Disc.Faraday Soc., 9, 461 (1950).

Walker, P.M.B. and Yates, H.B., Symp.Soc.exp.Biol., 6, 265 (1952a).

Walker, P.M.B. and Yates, H.B., Proc.Roy.Soc., London, 140B, 274 (1952b).

Wang, T.W., Kirkham, W.R., Dallam, R.D., Mayer, D.T. and Thomas, L.E., Nature, London, 165, 974 (1949).

Wang, T.W., Mayer, D.T. and Thomas, L.E., Exp.cell Research, 4, 102 (1953).

Warburg, O., Hoppe-Seyl.Z., 70, 413 (1910).

Watson, J.D. and Crick, F.H.C., Nature, London, 171, 737 (1953).

Weigert, O., Fortschr.Med., 2, 190 (1884).

Wieland, H. and Scheuing, O., Ber.d.d.chem.Ges., 54, 2527 (1921).

Wilkins, M.H.F. and Norris, K.P., Nature, London, 170, 884 (1952).

Williamson, M.B. and Gulick, A., J.cell.comp.Physiol., 20, 116 (1942).

Wyatt, G.R., Biochem.J., 48, 581 and 584 (1951).

Wyatt, G.R. and Cohen, S.S., Nature, London, 170, 1072 (1952).

Wyckoff, H., Lab.Investign., 1, 115 (1952).

Wyckoff, R.W.G., Ebeling, A.H. and Louw, T.A.L., J.Morphol., 53, 189 (1932).

Zeuthen, E., Pubbl.Staz.zool.Napoli, 23, 47 (1951).