THE MITOTIC CYCLE

THE CYTOPLASM AND NUCLEUS DURING INTERPHASE AND MITOSIS

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FOREWORD

EVEN at the risk of beginning with an unnecessary remark, it may be worth stating that this is not a text-book of cytology. The readers' acquaintance with the elements of the subject has been assumed. The intention of this book has been rather to review recent progress in the study of the dividing cell, and to relate it where possible to the history of the subject. It cannot be claimed that precisely equal justice is done to all branches and in common with most cytological works, a larger share of attention has been paid to those topics with which the researches of the author have been concerned. The centre of gravity lies more towards the physiological than to the descriptive aspects of the subject, though the attempt has been made, however imperfectly, to view the subject as a whole. In recent years there has been a marked growth of interest in the dividing cell, though for several reasons it is impossible yet to assess the extent of the permanent contributions to knowledge which are now being made. One cannot forecast for how long the present trends in research will be maintained, though their momentum is still high. Caution in any estimates of this kind is suggested by the previous reviews of this field, from which the contemporary reader may well have gained the impression that the way was clear for substantial progress in promising directions, though not always would such hopes have subsequently been fulfilled. For instance, in Professor FAURÉ-FREMIET's classical *Cinétique du Développement* the approach is from the standpoint of energetics, which has since been found more applicable to the wider embryological aspects of growth than to the study of the single cell in division. Again, Professor GRAY's *Experimental Cytology*, to which the writer in common with many others owe their interest in the problems of the cell, has the unifying theme of cell mechanics which is there expounded with masterly grace and penetration. It is a sobering experience to realize how little the past twenty years can add to the treatment of some topics on cell division to be found in that book. There can be no doubt that the single living cell in mitosis is one of the most difficult objects which the experimental biologist has ever attempted to analyse.

The present author has been fortunate in enlisting the help of two colleagues, Dr Charity WAYMOUTH and Dr Michael SWANN, each with special and indeed unique experience in their own fields; their contributions appear on pages 163-182 and 119-134 respectively. He is further indebted to them for the help which they have given in advice and criticism elsewhere in the book, though their responsibility is here entirely restricted to their own respective sections.

He is further indebted for much advice and assistance to Dr Honor Fell, Dr Michael Webb, and other colleagues at the Strangeways Laboratory; to Dr Gray and to Dr Pelc of the M.R.C. Radiotherapeutical Research Unit, to Dr C. D. Darlington and his colleagues at the John Innes Horticultural Institution, to Dr R. A. Beatty of the Department of Animal Genetics, Edinburgh, and to Dr Ivor Cornman of Washington who generously allowed him to make use of his unpublished survey of the effects of aromatic compounds on dividing cells, and to his publishers for their interest in this book and their promptitude in its publication.

Cambridge, October, 1951

'Les méthodes nouvelles de l'anatomie microscopique qui permettent de saisir la matière vivante aux differentes périodes de son évolution, de la fixer dans sa forme, de différencier, au moyen de réactifs chimiques, les éléments qui entrent dans sa constitution, les perfectionnements apportés aux objectifs des microscopes qui font apercevoir des détails qui devaient fatalement échapper aux anciens observateurs, ont montré que la structure et la vie d'une cellule sont plus complexes qu'on ne le pensait. Nous sommes loin aujourd'hui du temps où l'on considérait la cellule comme une petite masse de substance homogène, sarcode ou protoplasma, entourée ou non d'une membrane d'enveloppe et renfermant un petit corps réfringent, le noyau, contenant lui-même un corps plus petit, le nucléole.'

L. F. Henneguy, 1896.

'From the extensive investigations on the mechanism of cell division it must be concluded that there is as yet no full understanding of the physical and chemical mechanisms which bring forth this process.'

E. S. G. Barron, 1949.

'We have learned many things about cell division, but we do not know much in the end.'

W. D'Arcy Wentworth Thompson, 1942.

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THE NUCLEIC ACIDS

BEFORE we attempt to discuss the behaviour of the cell and of its microscopically appreciable components, there should be given some account of those macromolecules the changes in which, at a lower order of magnitude, are now known to relate to the visible events within the cell, and are believed in some way to initiate and govern the course of the whole complex of biological events which results in the production of two cells from one.

HISTORICAL

The history of this branch of biology is an interesting one, for it may be said that it began in the seventies and eighties of the last century with studies on the chemistry of the cell nucleus, which only in recent years have been resumed. Then, as now, there are two general methods which could be used for such investigations. Under the microscope one can study the behaviour of cells and tissues towards stains and reagents, or alternatively cell components such as nuclei can be separated from gross quantities of tissue and then analysed in bulk by chemical methods.

By the eighteen-sixties the affinity of cell nuclei for colouring agents such as carmine was well known (MANN,[1] BAKER[2]), and the recognition that the invariable presence of the nucleus pointed to its essential role in the life of a cell led MIESCHER, a pupil of HIS, to attempt its investigation by the second of these general methods, for which he needed a source of one type of cell in large quantity.* He first used pus from surgical bandages, plentiful in the pre-Listerian era, a material from which, although *nicht tadelfrei*, MIESCHER found that he could free the degenerating leucocytes, and after further trials could separate these as we should now say, into nuclear and cytoplasmic fractions. MIESCHER describes the isolated nuclei as 'vollkommen reinen Kernen, mit glatter Contour, homogenen Inhalt, sharf gezeichnetem Nucleolus, im Vergleich zur ihren ursprunglichen Volumen etwas verkleinert' (MIESCHER[5]). From these isolated nuclei, MIESCHER proceeded to prepare a substance which he termed 'nuclein' with stronger acidic properties than any organic cell constituent then known, and which was soluble in weak alkali, but not in dilute acid. It contained a high

* An admirable account of MIESCHER's life and work is given by GREENSTEIN.[3]

percentage of phosphorus; it was only very slowly attacked by gastric juice. An account of this research was submitted to HOPPÉ-SEYLER in 1869, but so startling were the nature of these conclusions that MIESCHER's[5] paper was not published until 1871, by which time other colleagues had confirmed these observations on different types of material. In that year, MIESCHER returned to his native city of Basle and became interested in the biology of the Rhine salmon, the sperm of which provided new material for investigations on nuclear chemistry. The acid nuclein of the sperm head was shown to be united with a nitrogenous base, to which MIESCHER gave the name protamine. He did not regard this as a protein, for it gave no reaction with the Millon reagent.

The first instance in which the results of MIESCHER were applied to a histochemical investigation is provided by the work of ZACHARIAS,[6] who sought to determine whether the nuclei of several types of cell consisted of nuclein, by testing if they were resistant to peptic digestion, and whether they would swell or gelate in strong sodium chloride, as did MIESCHER's nuclein. ZACHARIAS applied these tests to a number of animal and plant cells and found that the behaviour of their nuclei suggested that they also contained nuclein. This research FLEMMING[7] had in mind when in his classical *Zellsubstanz, Kern und Zelltheilung* he gave a definition of the substance which forms the 'framework' of the nucleus:

> which, in virtue of its refractile nature, its reactions, and above all, its affinity for dyes, is a substance which I have named chromatin. Possibly chromatin is identical with nuclein, but if not, it follows from ZACHARIAS' work that one carries the other. The word chromatin may serve until its chemical nature is known, and meanwhile stands for 'that substance in the cell nucleus which is readily stained'.

Although this definition was framed with respect to the resting nucleus, FLEMMING clearly describes in the following section of the book how the 'Kerngerüste' of the resting nucleus is directly transformed into the 'chromatischen Kernfigur' during mitosis, the individual loops of which were not given a special name until WALDEYER[8] first used the term 'chromosome'.

By the eighteen-seventies, there were available a number of the azo dyes which are still used by histologists, and FLEMMING in 1882 lists those which he had employed in studying the nucleus throughout its stages. It was not long afterwards that EHRLICH drew attention to the difference in staining properties between those dyes in which the colour group was acidic or basic (see for instance EHRLICH[9]).

Meanwhile, advances in the chemical study of the nucleus were in progress and the presence was being recognized of one type of constituent unit, the purine bases, then known as the 'alloxuric bases'. Such investigations were begun by PICCARD, and continued by KOSSEL. At

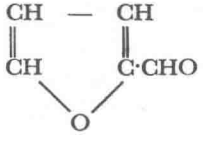
first it was not clear whether nuclein or protamine was the source of these bases. KOSSEL[10] had by 1881 inclined more to the former opinion, and continued to work on these substances from this point of view. In 1889, however, ALTMANN[11] first described a method for preparing nucleic acids which were free from protein, from both animal tissues and yeast, and within a few years KOSSEL and NEUMANN[12] were able to isolate thymonucleic acid from the thymus gland. These advances in the study of the structure of the nucleic acids did not lead to any immediate comparable progress in histochemistry. By means of double staining methods, LILIENFELD[13] attempted to distinguish between nucleoproteins and free nucleic acid in the nuclei of resting and dividing cells, but similar efforts by HEINE[14] led this author to the conclusion that such a distinction could not be made by the staining methods then available. When FISCHER[15] wrote his classical monograph on the *Fixierung, Färbung und Bau des Protoplasmas* the subject of biological staining was already in the complex state in which it has largely remained. In this work, he discussed the influence on the results obtained of such factors as the nature of the fixative, and the order in which stains are applied, both with tissue sections and with films of proteins and nucleic acids. It was recognized that methyl green has a special affinity for thymonucleic acid, although FISCHER pointed out that proteins, when fixed by heavy metal salts, can also absorb this stain. In a series of papers at this time (MANN[16]), the question was debated whether the affinity for dyes depends upon chemical factors or whether physical effects are alone involved. In the end, future progress depended not only on the further development of microchemical staining methods, but also on the use of specific enzyme preparations on tissue sections under the microscope. The first histochemical researches which related to the nucleoclastic enzymes were studies on autolysis. Such experiments indicate the presence of tissue ferments only when conducted in the presence of antiseptics, which SALKOWSKI[17] first used for this purpose. ZALESKI[18] demonstrated that purine bases were liberated on prolonged autolysis of plant material at 30° C. while OËS[19 20] showed that a few hours' incubation under these conditions was sufficient to break up and dissolve the chromosomes in dividing cells of both plants and animals.

The investigations of VAN HERWERDEN[21 22] differed in both methods and results. By means of the method described by SACHS[23] a nuclease was prepared from beef pancreas or spleen. The expressed juice of these organs was precipitated with saturated ammonium sulphate, and the washed product was subsequently dissolved in water and dialysed. Sections of echinoderm eggs were incubated for 24 hours in this preparation, and were then stained in haemalum. On comparing them with control sections incubated in water, VAN HERWERDEN found that

the basophil granules of the cytoplasm, the 'chromidia', had disappeared. These bodies must therefore be related in composition to nuclein. A similar treatment of sections of the intervertebral ganglia of the cat resulted in the dissolution of the Nissl granules. The demonstration that nucleic acid is found in the cytoplasm is thus nearly forty years old.

In VAN HERWERDEN'S experiments, the changes within the oocyte nucleus, either in the chromatin or the nucleolus were very much less marked. He returned to this subject in a further study (VAN HERWERDEN[22]) of the effects on the sperm head of both nuclease and 1 per cent hydrochloric acid. He found that in both fishes and echinoderms the basophily of the sperm nucleus was decreased after hydrolysis by the latter reagent in the cold, but that the mammalian sperm was resistant to this treatment. The same result was obtained in each case by digestion with nuclease. VAN HERWERDEN saw in his results an illustration of the observations of KOSSEL[24] and KOSSEL and EDLBACHER[25] that the linkage between the basic protein and nucleic acid was broken with particular ease in the sperm nucleoprotein of fishes and echinoderms. He further observed that the unripe mammalian sperm head was attacked by the agents which he employed.

We now know that it is the difference between the nucleic acids of the sperm head and of the egg cytoplasm which is primarily responsible for VAN HERWERDEN'S results. At the time, the conclusion was drawn from these results that the 'nuclease test' could not be used as a reliable index of the presence of chromatin. The verdict of WILSON[26] on this matter, still to be found in the third edition and its reprints is that 'it must be admitted, therefore, that we have no certain means of identifying "chromatin" in the cell apart from its morphological history'.



(i) Furfural

$CH_3—CO—CH_2—CH_2·COOH$

(ii) laevulinic acid

$HO—CH_2—CO·CH_2—CH_2·CHO$

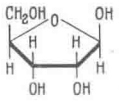
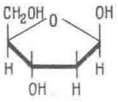
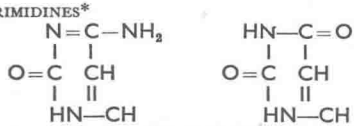
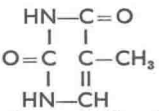
(iii) ω. hydroxy laevulinic aldehyde

Figure 1—STRUCTURAL FORMULAE

The recognition of two classes of nucleic acids preceded this cytochemical work of VAN HERWERDEN by many years, but until long after this period it was believed that one was found in plants and the other in animals. In 1893 KOSSEL[27] had shown that although nucleic acids from both yeast and animal sources contained the same purine bases, the

parent substances differ in that in the former there is a reducing carbohydrate which produces furfural on hydrolysis (Figure 1) and is thus a pentose. A further difference between the two groups is found in respect to the second category of constituent basic groups, the pyrimidines, the presence of which was recognized in 1893 by KOSSEL and NEUMANN.[28] In a series of papers published in 1903, LEVENE[29] showed that the pyrimidine cytosine was common to both plant and animal nucleic acids, but that uracil was only found in the former, and thymine only in the latter. MANDEL and LEVENE,[30] however, found uracil and not thymine in their analysis of the nucleic acid of fish eggs. In Table I the main points of difference between the two types of nucleic acid are summarized. The admirable reviews of SCHLENK[31] and DAVIDSON[32] should be consulted for further information in the chemical field.

TABLE I

	RNA	DNA
PENTOSE	 *d*-Ribofuranose	 2-Deoxy-*d*-ribofuranose
PYRIMIDINES*	 Cytosine and Uracil	 Cytosine and Thymine
NUCLEOPROTEIN WITHIN THE CELL	Cytoplasm, Nucleolus and Chromosomes	Chromosomes
TURNOVER RATE	high	low: varies with mitotic rate (FURST *et alii*[109])
MOLECULAR WEIGHT OF ISOLATED ACID	10,280–23,250 (yeast RNA, FLETCHER *et alii*[106])	500,000 (SIGNER *et alii*[107]) — 3,000,000 (GULLAND *et alii*[108])
READILY STAINED BY	Basic Dyes such as methylene blue and toluidine blue	Methyl Green and Schiff's base (after hydrolysis)

FEULGEN REACTION

The carbohydrate of thymonucleic acid, corresponding to the pentose of yeast nucleic acid was believed by Kossel and Neumann[28] to be a hexose, on the grounds that on hydrolysis laevulinic acid was formed,

*There have been several reports of the occurrence of 5-methyl cytosine in DNA's of different sources, the most recent of which is by WYATT.[110]

a known breakdown product of these sugars. This was believed for a number of years although certain observations did not accord therewith, notably the unstable nature of the sugar of thymonucleic acid, and the fact that elementary analysis gave an empirical formula for this acid which did not suggest that the molecule contained a hexose. In 1914, FEULGEN[33 34] drew attention to these discrepancies; three years later he showed that among the products of gentle hydrolysis is a substance which gives the Schiff's test for an aldehyde, namely the re-appearance of coloured fuchsin in a solution of dye decolorized by sulphur dioxide. This is the basis of the now famous Feulgen reaction, the application of which to microscopical preparations was subsequently described (FEULGEN and ROSSENBECK[35]). In this classical paper these authors apply the test to a number of tissues and organisms, including the wheat germ, from which triticonucleic acid had been prepared (OSBORNE and HARRIS[36]) similar in nature to that of yeast. They did not therefore expect to find, as they did, that sections of the wheat embryo gave a positive reaction with the test:

> This gave us a great surprise, for the nuclei of the wheat embryo gave the nucleal reaction more intensively than we have ever seen in any animal tissues. This was confirmed in other plants, and so it was demonstrated that the plant nucleus contains nucleal bodies. The old dualism of yeast and thymonucleic acids is thus set aside. . . .

It now seems remarkable that it took several years from the publication of this paper before biologists began to make use of the means which had been put into their hands to follow up this demonstration of a fundamental property common to both plant and animal nuclei. At the time, however, it was uncertain whether Feulgen's test related to the sugar of thymonucleic acid or was due to traces of furfural formed by hydrolysis of pentose nucleic acid (STEUDEL and PEISER[37]). Possibly, also there was confusion between Feulgen's nucleal reaction, and the 'plasmal' test for cytoplasmic aldehydes, liberated after a prolonged hydrolysis (FEULGEN and VOIT[38]), which also uses decolorized fuchsin as a reagent.

The proof that the carbohydrate of thymonucleic acid is indeed responsible for the Feulgen reaction was given by LEVENE and LONDON.[39] They were able to separate the constituent nucleosides, units consisting of base plus sugar, and later to isolate the latter component itself. LEVENE *et alii*[40] proved it to be *d*-2-deoxyribose. By this time, biologists had begun to use the Feulgen reaction (e.g. COWDRY[41]). The growth of the ensuing literature followed a sigmoidal curve; in 1938, MILOVIDOW[42] could list over 450 papers in this field. In recent years, discussion of the Feulgen reaction has centred round two points; one, whether the distribution of the liberated dye within a microscopical preparation indicates the precise localization of the DNA when the cell was alive;

secondly, if so, whether the reaction can be used quantitatively to measure this substance in absolute amounts.

All are agreed that aldehyde groups in the hydrolysis products of deoxyribose are responsible for the production of the purple colour; according to STACEY *et alii*,[43] it is *w*-hydroxy-laevulinic aldehyde which reacts with the fuchsin-sulphurous reagent (Figure 1). The question is whether the coloured substance formed by this reaction stays precisely at the site of formation, or whether it can diffuse into and stain neighbouring zones of the section. STEDMAN and STEDMAN[44] point out that this dye is water-soluble, but is readily adsorbed by chromosomin, the non-basic protein of the nucleus which these authors have discovered. The Feulgen reaction according to these authors indicates the position of the chromosomin stained by a dye liberated in a reaction in which deoxyribose took part.

The STEDMANS' argument is not demolished by the demonstration that a granule of DNA* when fixed, embedded in gelatin and sectioned, is Feulgen-positive (BRACHET[45]), for the liberated dye has then no chromosomin to which it may become attached. It would be useful to compare the result of this experiment with one in which a similar mass of DNA was embedded in gelatin to which chromosomin had been added. At the moment, the most cogent argument which can be brought forward to support the validity of the Feulgen reaction is the constancy of the results obtained when a standard procedure is adopted. For instance, the small Feulgen-positive heterochromatic granules of the interphase nucleus are seen in a constant relationship to the residual chromosome threads (FELL and HUGHES;[46] p 33). If in such instances there is diffusion of the liberated dye, it must be restricted to a radius little greater than the limits of resolution of the microscope.

The subject which we have just discussed is clearly antecedent to the further question of whether the Feulgen reaction can be made the basis of a quantitative microcolorimetric method. Several workers have assumed that all the necessary conditions are satisfied, and their results will be discussed in a further section (p 35). LESLER[47] has made a series of model experiments which relate to this question. He prepared a series of mixtures of gelatin with DNA at various concentrations, on which the Feulgen reaction was performed, and the results were judged subjectively. They did not suggest a uniform relationship between the concentration of DNA and the depth of colour produced.

Comparable debates on the validity of the methods used and the results obtained have occurred in other branches of cytochemistry. It must be borne in mind that the search for methods which will estimate

* In this book, the usual abbreviations of 'DNA' for deoxyribonucleic acid and 'RNA' for ribonucleic acid have been employed. The latter term is used loosely for all cytoplasmic nucleic acids; some authors prefer to speak of pentose nucleic acids (PNA), for it has not been proved that d-ribose occurs in them all.

or even recognize particular constituents within an area of a complex mixture of the order of a few square microns goes beyond the exactitude demanded of any analytical procedure elsewhere in microchemistry. However, rather than sit with folded hands until fully adequate methods are available, students of cellular biology have chosen to make use of the present inadequate procedures although the results obtained have often only a provisional status. To allow the tentative generalizations which emerge to harden into dogma by frequent reiteration is nevertheless an avoidable impediment to the development of the subject. Furthermore, it must be remembered that much of our knowledge of intracellular physiology is still well within the early phase of the development of a science at the qualitative level.

With these considerations in mind we must now turn to the discussion of two major developments in research relating to the cytochemical localization of the nucleic acids, namely the development of ultraviolet microspectrometry by CASPERSSON and his school, and secondly the isolation of separate nucleoclastic enzymes in approximate states of purity, exclusively affecting the two types of nucleic acid.

NUCLEOCLASTIC ENZYMES

It will be convenient here to discuss first the latter of these two developments. Following the usual nomenclature which uses the name of the appropriate substrate, these enzymes are now known as ribonuclease and deoxyribonuclease respectively. Both have been prepared from the pancreas, by which they are secreted for the digestion of nucleic acids liberated by the stomach acid from dietary nucleoproteins. The intracellular nucleases in other tissues are probably not identical with these pancreatic enzymes.

Ribonuclease proved much the easier enzyme to prepare, thanks to its remarkable stability towards both temperature and *p*H (JONES,[48] DUBOS and THOMPSON[49]). It was crystallized first by KUNITZ[50] in 1940, and found to be an albumen-like protein, with a molecular weight of approximately 15,000.

The method described by KUNITZ and NORTHROP[51] for the separation of chymo-trypsin from the pancreas was found to be of service also for the nucleases. The tissue is extracted in cold 0·25N sulphuric acid and by this means the activation of the tryptic enzymes is prevented. Ribonuclease is resistant to trypsin, but deoxyribonuclease is readily attacked thereby. From the filtered acid extract, the various enzyme proteins can be fractionally precipitated with different concentrations of ammonium sulphate; first deoxyribonuclease at a saturation of 0·4 and ribonuclease between 0·6 and 0·8. Deoxyribonuclease was prepared in this way by FISCHER *et alii*[52] in 1941, and by MCCARTY[53] in 1946.

It was crystallized by KUNITZ in 1948. MCCARTY developed a method for estimating the activity of a preparation of the enzyme, by making use of the high viscosity of sodium thymonucleate in solution. The rate of decrease is measured when the substrate is incubated with deoxyribonuclease. By this method, it was shown that the enzyme is activated by magnesium ions. One group of inhibitors are substances which remove this ion (Figure 2).

Numerous papers have been published on the cytochemical use of these enzymes. It is necessary to incubate a control series of sections in the buffer solution used to dissolve the enzyme, and then to compare the sections exposed to the latter with those treated with buffer alone. After ribonuclease digestion, either basophilia or absorption in ultra-

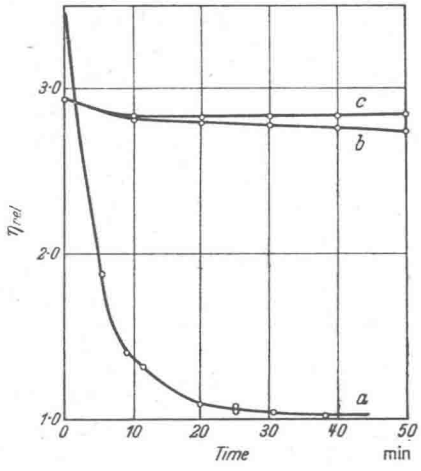


Figure 2 Relative viscosity curves of DNA **a** in the presence of desoxyribonuclease and 0·002 M $MgSO_4$ Magnesium, **b** with 0·002 M Sodium arsenate, **c** with 0·002 M Sodium citrate. (*By courtesy of Dr Michael Webb.*)

violet light is compared in the two series of preparations. Among the earliest papers in this field are those of BRACHET[54] in 1941, who used the methyl green-pyronin staining method of UNNA-PAPPENHEIM as the test for the effects of the enzyme. BRACHET found that in a number of cells and tissues, such as exocrine glands and oocytes in which there occurs a rapid synthesis of material, their basophilia was much reduced by treatment with ribonuclease.

DAVIDSON and WAYMOUTH showed that in the cytoplasm of rat liver cells, digestion with ribonuclease decreased both the affinity for toluidine blue[55] and the absorption of ultraviolet light.[56] In the nucleolus, the results were partly masked by the outer zone of heterochromatic deoxynucleoprotein. Although there are a number of apparently successful instances of the cytochemical use of ribonuclease, some authors including DAVIDSON[57] himself have drawn attention to difficulties in its use. STOWELL and ZORZOLI[58] have shown how much the

final results depend upon the nature of the fixative which was used, and conclude that 'with our present limited knowledge and technics, it would seem advisable to use the ribonuclease technic only crudely to confirm observations made by other methods'. DAVIDSON[57] believes that the main difficulty resides in the contamination even of the crystalline enzymes with traces of proteolytic ferments*. It has been suggested that certain inorganic reagents can remove ribonucleic acid from tissue sections more effectively than the enzyme. ERICKSON *et alii*[59] suggest perchloric acid for this purpose, while VENDRELY-RANDAVEL[60] and PONYET[61] advise the use of normal hydrochloric acid for ten minutes.

This difficulty of proteolytic contamination is even more marked with deoxyribonuclease, which does not share the heat-stable nature of the former enzyme, thanks to which it can be mainly freed of impurities. Several workers have added a very small amount of gelatin to their preparations of deoxyribonuclease which they have used for the digestion of sectioned biological material; WEBB and JACOBSON[62] have used small concentrations of tryptic inhibitors in the digestion mixture.

The extent to which deoxyribonucleic acid can be removed from the nucleus by digestion again depends among other factors upon the fixative originally used. Formalin should be avoided (STOWELL,[63] SANDERS[64]); chilled acetone is recommended by DAVIDSON.[65] Nuclei in the digested sections are sometimes still faintly Feulgen-positive (STOWELL, DAVIDSON, op. cit.), although a completely negative reaction following digestion is reported by CATCHESIDE and HOLMES[66] in the bands of salivary chromosomes, and by WEBB and JACOBSON[62] in smears of mouse and human cells. CATCHESIDE and HOLMES find that thymonuclease prepared from the spleen does not act on bean-root nuclei unless ribonuclease is used at the same time, while pancreatic deoxynuclease is effective alone.

DANIELLI[67] has criticized the use of these enzymes in cytochemistry with some severity, on the grounds of their probable impurity. He further suggests that a nuclease entirely free of proteolytic activity might fail to act if even a monolayer of protein surrounded the substrate within the tissue section. It is possible, however, that the recent successful use of nucleases in the presence of proteolytic inhibitors by WEBB and JACOBSON[62] have diminished the force of these criticisms.

But for the use of ribonuclease in cytochemistry there would have been no advance in the microscopical identification of ribonucleic acid subsequent to the recognition of its affinity for basic stains. Why such dyes as methylene and toluidine blue should be adsorbed more readily by RNA than by DNA is still not known, though it is surmised that free phosphate groups at the surface of the molecule are more

* McDONALD[4] has prepared ribonuclease free of all proteolytic activity.

abundant in the former type of nucleic acid. MICHAELIS[68] has shown that basic stains adsorbed on RNA are in the form of single molecules, but that the metachromatic staining of cartilage matrix, for instance, involves polymerization of the dye. In this interesting paper the author touches on a fundamental topic in this field. The living nucleus is not stained by methylene blue. When the stain is absorbed, the nucleus is dead; MICHAELIS believes that the dye has then displaced the protein from nucleic acid. In living cells are the whole nucleoproteins; their free components are produced by fixation. MONNÉ,[69] however, claims to have demonstrated a transient vital staining of the nucleus in *Amoeba* by micro-injecting dyes into the cytoplasm. These penetrated the nuclear membrane. The coloration within the nucleus soon disappeared; the cycle was more rapid with acid dyes than with basic.

SPECTROPHOTOMETRIC METHODS

Our final topic in the discussion of recent advances in the cytochemistry of the nucleic acids is the most important of the developments in this field, and is mainly responsible for the interest aroused in this subject in recent years. We refer to the development of spectrophotometric methods applied to the microscope, very largely by CASPERSSON and his school, which they use mainly in the ultraviolet range. At 2,600 Å, which corresponds to the resonance frequency of the pyrimidine and purine ring structure, the absorption of the nucleic acids is far more intense than that of other cell constituents in which these configurations are not found (Figure 3). The absorption band at this wavelength is equally a property not only of both DNA and RNA and their constituent nucleotide units, but also of the other adenylic acids and the co-enzymes. Thus ultraviolet techniques like any other cytochemical procedures are not alone sufficient to study the location of the nucleic acids within the cell.

A number of workers have demonstrated by photomicrography the presence of ultraviolet-absorbing substances within the cells and tissues; the special contribution of the Stockholm school is the development of quantitative methods by which, it is claimed, the extinction coefficient of an area one micron in diameter within a biological preparation can be measured to an accuracy of 1 per cent (CASPERSSON[70]). Such precision goes much beyond that obtainable by photographic methods; it is necessary to make direct photo-electric measurements through a range of wavelengths at a series of points within one single cell.

From a complete ultraviolet absorption curve, information can be obtained concerning the presence not only of nucleic acids but also of proteins which contain the aromatic amino acids, namely tyrosine, tryptophane, and phenylalanine. These show an absorption maximum