The Deoxyribonucleic Acid Content of the Cell Nucleus

by

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Note

Some of the experimental work described in Part II of this thesis was carried out in collaboration with Dr. F.C. Heagy, Dr. W.C. Hutchison and Dr. I. Leslie. The cell nuclei were isolated, counted and analysed by Dr. Heagy and the author, the counting and analyses being carried out in duplicate and independently. Dr. Hutchison and Dr. Leslie were responsible for the whole tissue analyses, with the exception of some of those shown in Table 24 which were performed by Dr. G.T. Mills and Dr. Evelyn E.B. Smith.

The cytophotometric experiments described in Part III were carried out in collaboration with Dr. S.C. Frazer. In general, Dr. Frazer was responsible for the staining and photomicrography, while the author undertook the preliminary isolation of the nuclei and made the actual measurements of area and optical density on the photographs obtained.

The author is entirely responsible for the statistical analyses included in Part II; the calculations required for the analyses of variance in Part III were carried out in collaboration with Dr. R.A. Robb and Dr. Frazer.

Contents

I.

Introduction

1.1 Historical.

It seems to be generally agreed that the word "cell" was first used in the biological sense by Robert Hooke in 1665 to describe microscopic structures which he observed in certain plant tissues and particularly in cork. Similar observations were made rather later in the same century by Malpighi, Leeuwenhoek and Grew, but after Leeuwenhoek's death in 1723 little further progress was made for nearly a century.

These early workers regarded cells as compartments into which the organism was divided; their attention was focussed on the cell wall rather than the cell contents, as is shown by the terminology which they used, viz., utriculi (Malpighi), cells and pores (Hooke), bladders (Grew). If they noticed the existence of the cell nucleus they did not attach any special significance to it. The modern view, that the lowest plants are single cells while higher plants are aggregates of cells, and that the nucleus plays a special rôle in the life of the cell, dates from the revival of interest in the microscopic structure of plants which took place at the beginning of the nineteenth century. It was first clearly and unambiguously stated by Schleiden in 1838, seven years after Brown had by his work

on Orchids drawn attention to the importance of the nucleus. In the following year Schwann extended Schleiden's theory to the animal kingdom and pointed out the essential similarity between plant and animal cells. Between 1840 and 1870 the fundamental outlines of the cell theory in something like its modern form were gradually worked out, the researches of Kölliker in embryology and Virchow in pathology being of particular significance. The cardinal importance of the nucleus was finally established in the last quarter of the century when Strasburger, Schneider, Flemming and others elucidated the complex process of mitosis and was further emphasized when Boveri, Sutton and others demonstrated the relationship between the chromosomes and the mechanism of Mendelian inheritance.

The first investigation of the chemical nature of the cell nucleus was undertaken by Friedrich Miescher in 1868. Pus cells obtained by extracting surgical bandages with a dilute solution of sodium sulphate were subjected to the action of artificial gastric juice. This treatment digested away the cytoplasm leaving the more resistant nuclei as an insoluble powder. If this powder was extracted with dilute sodium carbonate and the extract acidified with acetic acid a flocculent precipitate was obtained which was soluble in dilute alkali but insoluble in water, dilute acid or organic solvents. On examination, it was found to contain phosphorus

and to give the usual colour reactions for protein. Miescher called this material (which we now know to have been a nucleo-protein) "nuclein". Shortly afterwards Hoppe-Seyler and his associates obtained similar substances by applying Miescher's methods to yeast and to erythrocytes. In his later work Miescher used as a readily accessible source of "nuclein" the spermatozoa of the Rhine salmon. From this starting material he was able to isolate a protein-free "nuclein" (i.e., a nucleic acid) and a nitrogen-rich organic base of unknown constitution which he named "protamine" (Miescher,1897).

1.2 The structure of the nucleic acids.

Research into the occurrence and properties of the nucleic acids was first put on a firm basis when Kossel (1879 - 1888) discovered that they contained so-called "alloxuric bases" (in modern terminology, purine bases) and could thereby be distinguished both from proteins and from other naturally-occurring phosphorus compounds. Progress was, however, still impeded by the lack of a satisfactory general method for the preparation of protein-free nucleic acids. This was supplied when in 1898 Neumann, disregarding Miescher's warning about the possible consequences of using high temperatures and prolonged treatment with alkali, devised a method based on the extraction of minced tissue with hot dilute sodium hydroxide. It is no exaggeration to say that the use of this and other similar isolation techniques profoundly

influenced the future course of nucleic acid research. The tacit assumption that nucleic acids were capable of withstanding such drastic treatment unchanged led ultimately to a totally false impression of their structure and possible functions. In the meantime, however, the difficulties facing the analyst were considerably reduced and the next thirty years saw a lengthy and laborious investigation by the classical methods of organic chemistry of the detailed structure of the nucleic acid molecule, an investigation with which the names of Kossel, Hammarsten, Mandel, Levene, Jorpes, Steudel, Jones and Feulgen are especially associated. In the early years of the present century it was established that nucleic acids of plant and animal origin contained only two purine bases, adenine and guanine, in roughly equimolecular proportions. At about the same time it was shown that the nucleic acids isolated from animal tissues contained, in addition, the pyrimidine bases cytosine and thymine, whereas those isolated from yeast and wheat embryo contained cytosine and uracil (Jones, 1920a). In 1909 Levene identified the nitrogen-free organic component of yeast nucleic acid as D(-)ribose and twenty years later the same author showed that the corresponding component of thymus nucleic acid was D-2-deoxyribose. The manner in which these units were linked to one another and to the phosphorus present in the molecule was made clear when nucleosides (condensation

products of a purine or pyrimidine with a pentose or deoxypentose) and nucleotides (phosphoric esters of nucleosides) were discovered in the hydrolysis products of both yeast nucleic acid and thymus nucleic acid.

These chemical studies were comprehensively reviewed by Levene & Bass in 1931. By that time it had become apparent that nucleic acids were polynucleotides and that they could be divided into two classes. One class, the so-called ribonucleic acids (RNA), typified by yeast nucleic acid, contained adenine, guanine, cytosine and uracil together with a pentose, known in the case of yeast RNA to be D(-)ribose. The other class, the deoxyribonucleic acids (DNA), of which thymus nucleic acid was the member most thoroughly investigated, contained adenine, guanine, cytosine and thymine together with a deoxypentose, known in the case of thymus DNA to be D-2-deoxyribose. (It has been pointed out by Davidson, 1950, that the use of the words "ribonucleic" and "deoxyribonucleic" to describe nucleic acids other than those which have been positively shown to contain ribose and deoxyribose, respectively, is, strictly speaking, unjustified. In accordance with convention, however, the abbreviations RNA and DNA will be used throughout the present work as generic terms to denote pentose and deoxypentose nucleic acids, respectively.)

Since both RNA and DNA had been shown to contain four

nucleotides and since there was some evidence that these were present in equimolecular proportions the early workers believed that both types of nucleic acid had very probably a tetranucleotide structure (see figs. 1 and 2).

Onee it was accepted that the nucleic acids had a structure of this comparatively simple sort the question naturally arose whether all specimens of RNA, irrespective of the tissue from which they were obtained, might not be identical with yeast RNA, and all specimens of DNA similarly identical with thymus DNA. Such evidence as was available seemed to suggest that this was, in fact, the case. The wheat germ RNA of Osborne & Harris (1902) had properties very similar to those of yeast RNA and Jones (1908) was unable, in the course of an optical rotation study, to find significant differences between the DNAs of thymus, pancreas and spleen. It is not therefore surprising that Jones (1920a) should have categorically stated that there were only two nucleic acids or even that Levene & Jacobs (1912) should have published a paper on the structure of "thymus nucleic acid" based entirely on results obtained with fish sperm DNA.

The earliest attempts to estimate the molecular weight of nucleic acids were made by Myrback & Jorpes (1935). The figures of 1300 - 1700 reported by these workers were, allowing for the errors and uncertainties of their method,

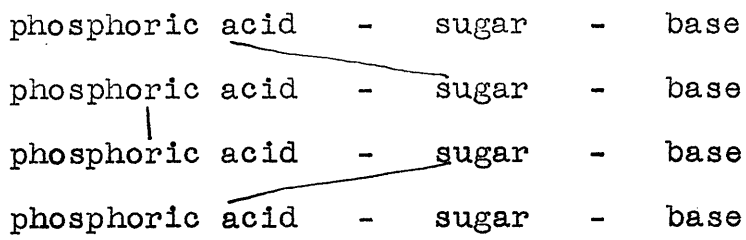

Figure 1.

Tetranucleotide structure proposed for nucleic acid by Feulgen (1918).

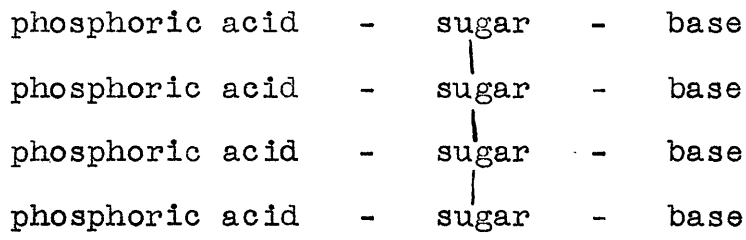

Figure 2.

Tetranucleotide structure proposed for nucleic acid by Jones (1920b).

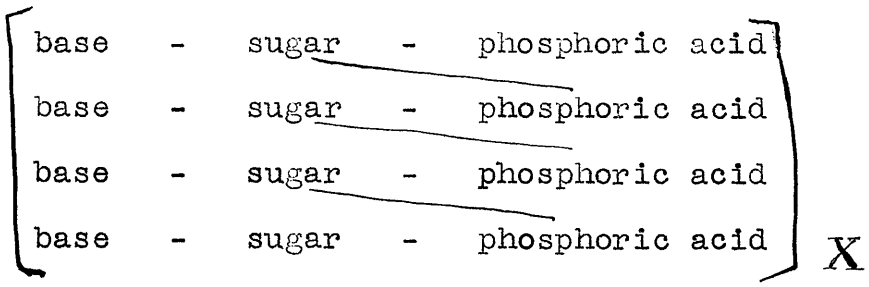

Figure 3.

Polytetranucleotide structure proposed for nucleic acid (Greenstein, 1944).

Table 1.

Estimates of the molecular weights of ribonucleic acids (RNA) prepared from various sources.

Source	Molecular weight	Reference
	16,300 - 23,900	(1)
Yeast	17,000	(2)
	10,280 - 23,250	(3)
Escherichia coli	17,500	(1)
Tobacco mosaic	37,000	(2)
virus	50,000 - 300,000	(4)
Pancreas	11,500	(1)

from (1) Delcambe & Desreux (1950),

(2) Loring (1939),

(3) Fletcher, Gulland, Jordan & Dibben (1944),

and (4) Cohen & Stanley (1942).

Table 2.

Estimates of the molecular weight of calf thymus deoxyribonucleic acid (DNA).

Method	Molecular weight	Reference
Sedimentation and diffusion measurements	1,500,000	(1)
	1,500,000	(2)
	500,000	(3)
	820,000	(4)
Measurements of light scattering	3,700,000	(5)
Dielectric measurements	35,000 - 135,000	(6)

from (1) Kahler (1948),

(2) Conway, Gilbert & Butler (1950),

(3) Tennant & Vilbrandt (1943), Vilbrandt & Tennant (1943),

(4) Cecil & Ogston (1948),

(5) Smith & Scheffer (1950),

and (6) Jungner, Jungner & Allgen (1949), Jungner (1950).

in reasonably good agreement with the theoretical molecular weight of a tetranucleotide (1357). These results were, however, obtained from material isolated and purified by a fairly drastic technique.* When determinations were carried out on RNA and DNA isolated by milder procedures much higher figures were obtained (see Tables 1 and 2). It is perhaps unfortunate that the results obtained by different workers using different methods should vary so widely. Nevertheless even if the lowest estimates of molecular weight shown in Tables 1 and 2 are assumed to be correct it follows that the RNA molecule must contain at least 30 nucleotide units and the DNA molecule at least 100.

Curiously enough, the discovery that the nucleic acid molecule contained more than 4 nucleotide units did not lead to the complete abandonment of the tetranucleotide theory. Instead, it was suggested that the nucleic acid molecule might be, not a single tetranucleotide, but a polymer or polycondensation product in which a tetranucleotide was the repeating unit. The formula shown in fig.3, which was first proposed by Levene (Levene & London, 1929; Levene & Bass, 1931), was regarded as late as 1944 as representing the probable structure of both RNA and DNA (Greenstein, 1944).

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*The methods of Myrback & Jorpes (1935) have been severely criticised by Fletcher (1948) who has recalculated their results and obtained a value of 6000 for the molecular weight of their material.

It was left to Gulland and his associates to point out how weak the experimental foundation for this "polytetranucleotide" hypothesis really was (Gulland, Barker & Jordan, 1945; Gulland, 1947a, b; Creeth, Gulland & Jordan, 1947). It depended entirely on the assumption that the four nucleotides of a nucleic acid were present in equimolecular proportions. A considerable body of evidence indicated that this assumption was at least approximately true and so long as the nucleic acid molecule was thought to be small (e.g., containing 4 or 8 nucleotides) minor discrepancies between the actual results of analyses and the theoretical requirements of the tetranucleotide formula could be attributed to experimental error. When it became clear that the nucleic acid molecule contained at least 30 nucleotides this practice ceased to be permissible. But even if it is assumed that the four nucleotides are present in exactly equimolecular proportions it by no means follows that they must necessarily be arranged in a regular unchanging sequence thus:-

ABCDABCDABCDABCDABCDABCDABCD.

They may equally well be arranged in a quite irregular manner thus:-

DACCCADBDADDCBCCBAADACAB.

Gulland called this sort of structure a statistical, as opposed to a structural, tetranucleotide.

Even in 1945 there was some evidence that the four

nucleotides were not present in exactly equimolecular proportions in all nucleic acids. For example, the RNA of tobacco mosaic virus had a purine content 20% lower than that predicted by the tetranucleotide theory (Loring, 1939); pancreas RNA is recorded as having a considerable, if variable, excess of guanylic acid (Jorpes, 1928, 1934; Levene & Jorpes, 1930); the RNA of rat liver has a nitrogen: phosphorus ratio higher than that required by the theory (Brues, Tracy & Cohn, 1944); and Gulland himself was able to show that the purine nitrogen : pyrimidine nitrogen ratios of yeast RNA and calf thymus DNA were 1.86 and 1.60 respectively instead of 2.0 as the theory would indicate (Gulland, Jordan & Threlfall, 1947).

In recent years the application of paper chromatography to the separation of hydrolysis products and the estimation of these products by ultraviolet absorption measurements have greatly facilitated the investigation of the nucleotide composition of the nucleic acids (Hotchkiss, 1948; Vischer & Chargaff, 1948; Markham & Smith, 1949; Smith & Markham, 1950). The results obtained by these methods have been quite inconsistent with the tetranucleotide theory in any of its various modifications. In particular, the use of chromatography has led to the discovery, among the hydrolysis products of herring sperm DNA, of a fifth nitrogenous base, the aminopyrimidine 5-methylcytosine (Wyatt, 1950).

The existence of the corresponding nucleotide was subsequently demonstrated by Cohn (1951b). It appears to occur in the DNA of all higher plants and animals so far investigated but, except in the case of wheat germ DNA, it is present only in very small amounts. It has not so far been detected in the DNA of bacteria or viruses or in RNA (Wyatt, 1951). Quite recently Wyatt & Cohen (1952) have reported the occurrence, in the DNA of bacteriophage, of a sixth base thought to be hydroxymethylcytosine. It would therefore be unwise to assume that no new components of nucleic acids remain to be discovered.

A further complication was introduced into the problem of nucleic acid structure when Carter & Cohn (1949) demonstrated that in hydrolysates of RNA there exist 2 adenylic acids separable on ion exchange columns (Carter & Cohn, 1949) or by paper chromatography (Carter, 1950). It was subsequently shown that 2 guanylic acids (Cohn, 1949b, 1950a; Volkin & Carter, 1951), 2 uridylic acids and 2 cytidylic acids (Cohn, 1950b) are also present in RNA hydrolysates. It is not yet known whether all 8 nucleotides are actually present in the RNA molecule. Since in each case the two isomers are readily interconvertible in hot mineral acid it is possible that one might be formed from the other during the process of hydrolysis (Cohn, 1951a). No similar isomerism has yet been reported in the nucleotides of DNA.

Table 3.

Nucleotide composition of ribonucleic acid (RNA) from various sources. Results expressed as moles guanylic acid, cytidylic acid and uridylic acid per 10 moles adenylic acid.

Source	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid
Yeast	10	9.7	6.1	7.0
Pig pancreas	10	22.5	9.8	4.6
Pig liver	10	16.3	16.1	7.7
Sheep liver	10	16.8	13.4	5.6
Calf liver	10	16.2	11.1	5.3
Beef liver	10	14.6	10.9	6.6

from Chargaff, Magasanik, Vischer, Green, Doniger & Elson (1950).

Table 4.

Nucleotide composition of deoxyribonucleic acid (DNA) from various sources. Molar ratios calculated to a total of 4.00

Source	Adenine	Thymine	Guanine	Cytosine	5-Methyl-cytosine
Calf thymus	1.13	1.11	0.86	0.85	0.054
Beef spleen	1.13	1.12	0.85	0.85	0.054
Bull sperm	1.15	1.09	0.89	0.83	0.052
Ram sperm	1.15	1.09	0.88	0.84	0.039
Herring sperm	1.11	1.10	0.89	0.83	0.075
Wheat germ	1.05	1.08	0.94	0.69	0.23
Bovine tubercle bacilli	0.71	0.76	1.17	1.35	0
Bacteriophage T_5	1.34	1.44	0.82	0.41	0

from Wyatt (1951) and Smith & Wyatt (1951).

Table 5.

Approximate "nucleotide formulae" and minimum molecular weights of deoxyribonucleic acids (DNA) from various sources.

Source	Relative numbers of nucleotides						Minimum molecular weight
	Adenine	Thymine	Guanine	Cytosine	5-Methyl-cytosine	Total	
Beef spleen	21	21	16	16	1	75	23,000
Herring sperm	15	15	12	11	1	54	17,000
Wheat germ	9	9	8	6	2	34	11,000

from Wyatt (1952).

Table 6.

Nucleotide composition of nuclear and cytoplasmic ribonucleic acids (RNA) from various calf tissues. Results expressed as moles guanine, cytosine and thymine per 10 moles adenine.

	Adenine	Guanine	Cytosine	Thymine
Nuclear RNA				
Liver	10	10.0	3.9	4.5
Thymus	10	8.8	0.9	1.7
Kidney	10	9.2	2.5	5.9
Heart	10	9.2	1.9	2.9
Cytoplasmic RNA				
Liver	10	18.3	12.2	11.2
Thymus	10	18.8	14.4	8.9
Kidney	10	17.8	12.5	9.4
Heart	10	12.0	7.7	0.3

from Marshak (1951).

Some of the results obtained for the composition of RNA and DNA from various sources are shown in Tables 3 and 4 respectively. In general, it appears that the composition of RNA varies from one organ to another while the composition of DNA is fairly constant for the different tissues of any one species (Chargaff, 1950). So far as adenine, guanine, cytosine and thymine are concerned there is remarkably little difference in composition between the DNAs of different animal species. The content of 5-methylcytosine, on the other hand, varies markedly from one species to another (Wyatt, 1952). It has been pointed out by Chargaff (1951) that there is a remarkable constancy in the ratios of adenine : thymine and guanine : cytosine among DNAs from different sources. The fact that, in animal DNAs at least, both ratios approximate to unity (see Table 4) does suggest that DNA might perhaps be built up from adenine-thymine and guanine-cytosine dinucleotides. It would, however, be most unwise to place too much emphasis on this point until more information is available. Chargaff, Zamenhof, Brauerman & Kerin (1950) have also drawn attention to the fact that the DNAs so far investigated fall into two types: an "AT" type in which adenine and thymine predominate and which includes all animal DNAs and some also of microbial origin; and a "GC" type found in some micro-organisms in which guanine and cytosine predominate. These observations

have been confirmed by Smith & Wyatt (1951) and Wyatt (1952). Their chemical and biological significance is a matter for speculation.

Just as in classical organic chemistry the empirical formula and minimum molecular weight of a compound may be calculated from its elementary analysis, so also the "nucleotide formula" and minimum molecular weight of a nucleic acid may be calculated from its nucleotide analysis. A number of such "nucleotide formulae", calculated by Wyatt (1952) for DNAs from various sources, are shown in Table 5. The validity of such calculations rests, of course, on the assumption, which until quite recently seems to have been tacitly accepted by most workers in this field, that the DNA (or RNA) isolated from a tissue consists of a single molecular species. Recent work has suggested that this is not, in fact, the case. Stern & Atlas (unpublished experiments quoted by Stern, 1952) have found all the DNA preparations which they have so far examined to be polydisperse. Bendich (1952) has presented evidence that several rat tissues contain at least two DNAs with different metabolic activities. Barton (1952) has shown that isolated cell nuclei contain two DNA fractions, one of which is resistant to the action of the enzyme deoxyribonuclease. The uniformity in nucleotide composition of the DNAs isolated from different tissues of the same species, which was referred to

above, seems to suggest that the heterogeneity disclosed by these observations is due, not to the occurrence of different DNAs in the different types of cell within a single tissue, but rather to the occurrence of two or more distinct types of DNA within each individual cell. Alternatively, it might be postulated that all the molecules of DNA in an organism have the same structure, which is presumably specific for the species, but that during isolation or under treatment with deoxyribonuclease these giant molecules break down to give fragments of varying chemical and physical properties. In this connection it is of interest to recall that Zamenhof & Chargaff (1949) have demonstrated that isolated DNA contains a "core" which differs from the rest of the molecule in its chemical composition and its resistance to deoxyribonuclease.

The heterogeneity of RNA is much better understood. It has already been pointed out that the composition of RNA varies from one tissue to another (Chargaff, 1950). Probably, therefore, it also varies from one cell type to another within the same tissue. There is evidence also for the existence of at least two types of RNA within the same cell. Thus several workers have shown that in a variety of tissues the RNA of the isolated nuclei has a different composition from that of the cytoplasm (Chargaff, 1950; Marshak, 1951). Marshak's (1951) results are of particular

interest since they indicate that there are differences in composition in the nuclear as well as the cytoplasmic RNAs of different tissues of the same species (see Table 6). McIndoe & Davidson (1952) have reported similar differences in the composition of nuclear RNA.

These purely chemical studies have obviously a most important bearing on the question of the biological significance of DNA and RNA. If the nucleic acid molecule was simply a tetranucleotide or a polytetranucleotide and if all RNAs and all DNAs were identical it would be necessary to assume that the nucleic acids performed some simple function, or functions, comparable perhaps to those of the polysaccharides. If, on the other hand, the constituent nucleotides of the molecule are not arranged in a regular sequence of simple repeating units the possibilities of isomerism are enormously increased and it becomes possible to think of nucleic acids as being possessed of the same degree of specificity as proteins (Gulland, 1947a, b) and perhaps fulfilling functions equally varied and specialised. It might reasonably be expected that X-ray studies would throw some light on this crucial question of "regularity" within the molecule. Astbury (Astbury & Bell, 1938; Astbury, 1947) has indeed concluded from studies of this sort that there are well-marked periodicities in the DNA molecule and more recently Riley & Oster (1951) have drawn similar conclusions. It appears, however, that the X-ray methods

available are not capable of distinguishing between nucleotides containing different bases and the results obtained by their use need not, therefore, be taken as indicating that DNA is built up from a small oligonucleotide repeating unit.

1.3 The localization of the nucleic acids.

Up to about 1930 research on nucleic acids was directed almost entirely to the elucidation of their detailed structure. Their function and their biological properties (as distinct from their catabolism) were virtually ignored. Nevertheless, the early workers did at least discover that the materials which they studied could be isolated from many different sources. Levene & Bass (1931), for example, listed in their monograph 19 tissues from which DNA could be obtained. Since almost all the nucleic acids isolated from animal tissues were of the DNA type and the only two plant nucleic acids extensively studied (those obtained from yeast and from wheat germ) were of the RNA type it was concluded, in the words of Jones (1920a), "that there are but two nucleic acids in nature, one obtainable from the nuclei of animal cells, and the other from the nuclei of plant cells." It does not seem to have been generally realised that the only reason for assuming that the nucleic acids were confined to the nucleus was that Miescher's early experiments had been carried out on isolated pus cell nuclei and sperm heads.

There was, even in 1920, some evidence that the situation might not be quite so simple as Jones thought. In 1894 Hammarsten had isolated from pancreas a nucleoprotein which he showed to contain guanine and a pentose. Similar materials were subsequently obtained from spleen by Jones & Rowntree (1908) and from liver by Levene & Mandel (1908). Since, however, these so-called β-nucleoproteins were not shown to contain more than one of the four nitrogenous bases they were not regarded as containing true nucleic acids. The first real demonstration of the occurrence of RNA in animal tissues was due to Jones & Perkins (1924 - 25) who showed that the hydrolysis products of pancreas β-nucleoprotein included adenylic, guanylic and cytidylic acids (see also Jorpes, 1928). Subsequent work has shown that RNA is universally distributed among animal as well as plant cells (Davidson, 1947a, b, 1950).

The occurrence of DNA in plant tissues was demonstrated by very different methods. For many years the fact that Schiff's reagent (fuchsin decolourised with sulphurous acid) gives a red colour with aldehydes has been known to organic chemists. In 1914 Feulgen found that after mild hydrolysis with N hydrochloric acid DNA, but not RNA, also gave a red colour with fuchsin-sulphurous acid. By applying this test to histological specimens Feulgen & Rossenbeck (1924) were able to demonstrate the presence of DNA in the nuclei of

wheat embryo cells. By itself, this observation could hardly be regarded as conclusive but thirteen years later Feulgen, Behrens & Mahdihassan (1937) separated dry, very finely pulverised rye embryo cells into nuclear and cytoplasmic fractions by the method of Behrens (1932) and isolated from the nuclear fraction a nucleic acid of the DNA type. In the following year Behrens (1938) again separated rye embryo cells into nuclear and cytoplasmic fractions and on this occasion isolated a nucleic acid of the RNA type from the cytoplasmic fraction. These experiments suggested that the real distinction between DNA and RNA was that the former was confined to the nucleus while the latter was present in the cytoplasm. The truth of this hypothesis has been repeatedly confirmed by cytochemical methods (Caspersson, 1936, 1939a, b; Caspersson & Schultz, 1938, 1939, 1940; Caspersson, Nystrom & Santesson, 1941; Brachet, 1940a, b, 1942) and, more recently, by experiments in which tissues have been separated into nuclear and cytoplasmic fractions (Schneider, 1946a, b, 1948; Hogeboom, Schneider & Pallade, 1948; Schneider & Hogeboom, 1950; Le Page & Schneider, 1948; Schneider & Potter, 1949; Schneider, Hogeboom & Ross, 1950; Price, Miller & Miller, 1948; Price, Miller, Miller & Weber, 1950; Petermann, Alfin-Slater & Larack, 1949). The presence of DNA outside the nucleus has been reported in only a small number of special cases: in certain plant cells

(Sparrow & Hammond, 1947); in Paramecium known to contain the cytoplasmic killer factor "kappa" (Preer, 1948); and in egg white (Fraenkel-Conrat & Ducay, 1951).

Quite apart from the theoretical significance of the results which they obtained the work of Feulgen and Behrens is of interest in that it illustrates the two main approaches by which the biochemistry of the cell nucleus may be investigated, namely,

(i) the identification and localization of a particular chemical compound in a histological tissue section, and

(ii) the isolation of nuclei from a tissue in such large numbers as to allow of their being submitted to gross chemical analysis.

Both of these methods require discussion in some detail. The isolation of nuclei will be considered first.

1.4 The isolation of cell nuclei.

It has already been said that Miescher originally discovered nucleoproteins in nuclei which he isolated from pus cells by the action of artificial gastric juice. This appears to have been the first occasion on which nuclei were isolated for the purposes of research although, according to Vendrely (1952), a method for isolating nuclei from tumour tissue using acetic acid was described in a textbook of microscopy as early as 1856 (Smith, 1856).

Miescher's practice of treating spermatozoa with dilute acetic acid in order to isolate their heads might perhaps be regarded, in a sense, as an isolation of sperm nuclei (Miescher, 1897). The scanty cytoplasm of the spermatozoon makes it, in some respects, particularly suitable for nuclear investigations and, among the earlier workers, Mathews (1897), Steudel (1913) and Lynch (1920) all published analyses of whole sperm or sperm heads. More recently Zittle and his associates have disintegrated bull spermatozoa into heads, midpieces and tails by using a magnetostriction sonic oscillator, a method originally used by Chambers & Flosdorf (1937) to disrupt bacteria and subsequently applied to spermatozoa by Henle, Henle & Chambers (1938). The sperm heads were then isolated by differential centrifugation. Zittle has analysed the material obtained in this way (Zittle & O'Dell, 1941) and investigated its content of cytochrome oxidase (Zittle & Zitin, 1942a) and of iron (Zittle & Zitin, 1942b). A certain amount of work on the nature of the proteins of the nucleus has also been carried out on sperm (Stedman & Stedman, 1943a,b; 1947a,b; 1951; Pollister & Mirsky, 1946; Daly, Mirsky & Ris, 1951).

The other important source of nuclear material used by the earlier workers was the nucleated avian erythrocyte. A wide variety of methods have been used to disrupt the erythrocyte membrane. Ackermann (1904) produced lysis by

the action of distilled water. Warburg (1910) achieved the same result by quick freezing, followed by thawing to 30°C. This method seems also to have been used by Miyake (1933). Later workers have, however, generally preferred to use a detergent, e.g., saponin (Yakusizi, 1936; Dounce & Lan, 1943; Stedman & Stedman, 1951), lysolecithin (Laskowski, 1942, 1943), tyrothricin (Villela, 1947), or cetavlon (Stedman & Stedman, 1951). When laking is complete the free nuclei may be centrifuged down and washed free of cytoplasmic contamination.

Spermatozoa and nucleated erythrocytes are unfortunately too highly specialised in their respective functions to allow results obtained from them to be used as the basis of any general theory of the function of the nucleus, and for many years nuclear research was gravely impeded by the lack of a satisfactory method for the isolation of nuclei from compact tissues. The first such method to be devised was that of Behrens (1932). Essentially it consists of freezing the tissue hard immediately after removal from the animal, dehydrating it while still in the frozen state, and liberating the nuclei by grinding the dried tissue very finely. The nuclei are then separated from the cytoplasm by centrifuging the resultant powder in a mixture of benzene and carbon tetrachloride, the specific gravity of which has been so adjusted that while the nuclei sediment the cyto-

plasmic material, which is slightly less dense, floats upwards and forms a crust on the surface of the suspending medium. This method is capable of very general application; Behrens himself used it to isolate nuclei from heart muscle (Behrens, 1932), haemosiderin granules from spleen (Behrens, 1933), colloid from thyroid gland (Behrens, 1935), nuclei from rye germ (Feulgen, Behrens & Mahdihassan, 1937), cytoplasm from rye germ (Behrens, 1938) and nuclei from liver (Behrens, 1939). It also has the advantages that the tissue undergoes a virtual fixation during the initial freeze-drying and that, since the suspending media used are immiscible with water, there is no danger of water-soluble constituents being dissolved out of the nuclei during the isolation process. Lipid material, on the other hand, will be lost but since comparatively few compounds of biological interest fall within this class (in comparison, that is, with the numerous class of biologically important water-soluble compounds) and since these may be readily studied in nuclei isolated by other methods, this is not perhaps a very grave defect. A more serious drawback is that the entire procedure is exceedingly laborious and time-consuming. (The time required to isolate nuclei from a single tissue may be measured in days and even weeks rather than hours.) The method is therefore unsuitable for routine use and is indeed seldom employed at all e xcept where

the usual techniques of isolation in aqueous media are inapplicable. It has been used within recent years in an investigation of the proteins, enzymes and free amino-acids of the nucleus by Dounce, Tishkoff, Barnett & Freer (1950). Allfrey, Stern, Mirsky & Saetren (1952) in their studies on nuclear enzymes have employed a modification of the method in which cyclohexane is used in place of benzene. They claim that in this way inactivation of enzymes during the isolation process may be reduced. It should also be pointed out that the process of grinding the dried tissue inevitably damages some of the nuclei (see photographs of Dounce et al., 1950 and Allfrey et al., 1952). The product, therefore, may be regarded as consisting of nuclear material rather than intact isolated nuclei.

It has already been remarked that in 1856 Smith described a method for the isolation of cell nuclei using acetic acid. Smith's work seems, however, to have attracted little attention and his method remained virtually unknown for nearly a century. The credit for re-discovering the fact that nuclei can be obtained from a compact tissue by treatment with a dilute aqueous solution of an organic acid must be accorded to Crossmon (1937) who reported that by immersing a small piece of muscle in a drop of 5% citric acid on a microscope slide some of the nuclei could be liberated from the tissue. The muscle fragment might then

be removed with forceps and the isolated nuclei stained in the usual way. Two years later Stoneburg (1939) used citric acid to isolate nuclei in bulk from muscle and tumour tissue. Stoneburg's method is of some academic interest in that he revived Miescher's practice of using pepsin and hydrochloric acid to digest away unwanted cytoplasm. In 1941 Marshak described a method for the isolation of nuclei in bulk from liver (a much easier process than isolation from muscle) again using citric acid but without the aid of pepsin or hydrochloric acid. This method, which, in its various modifications, is probably the most popular, and certainly the easiest, isolation technique, consists essentially of two operations.

(i) The tissue is minced and either homogenized or vigorously stirred in dilute citric acid (5% citric acid in Marshak's original method), which appears to have the effect of dissolving some of the cytoplasm while rendering the nuclei more resistant to mechanical damage. Mirsky & Ris (1946) have observed that nuclei isolated in citric acid withstand prolonged runs in the Waring blendor while untreated nuclei are disrupted within 4 - 15 minutes.

(ii) The homogenate is filtered to remove fragments of tissue which have escaped disintegration in the

homogenizer and is then centrifuged so that most of the nuclei, together with some cytoplasmic material, are spun down. The nuclei may be freed of cytoplasmic contamination by repeatedly resuspending in dilute citric and centrifuging down.

A number of modifications of this method have been devised particularly in respect of the technique of homogenization and the precise composition of the dilute organic acid used as isolating medium (Haven & Levy, 1942; Mirsky & Pollister, 1946; Vendrely & Vendrely, 1948; Barnum, Nash, Jennings, Nygaard & Vermund, 1950; Stedman & Stedman, 1950).

The citric acid method is very much less laborious and time-consuming than that devised by Behrens. Unfortunately the use of an aqueous isolation medium introduces the possibility that water-soluble material, including proteins, may be lost from the nuclei during isolation and the low pH employed must be expected to denature at least some of those proteins which remain. A solution to the latter problem has been found by Dounce (1943a, 1950, 1952a) who has devised a method of isolation in which citric acid is used only to lower the pH to 5.8 - 6.0. Nuclei isolated by this technique have been successfully used by Dounce and his associates in their studies on nuclear enzymes (Lan, 1943, 1944; Dounce, 1943a, b, 1950a, b; Dounce & Beyer, 1948).

A number of other aqueous isolation media have also been employed. Arnesen, Goldsmith & Dulaney (1949) use 0.25M sucrose dissolved in 0.008M citric acid for the isolation of nuclei from mouse spleen. For the same purpose Schneider & Petermann (1950) use 0.88M sucrose containing a trace (0.0018M) of calcium chloride, which is said to prevent disruption of the nuclei. Wilbur & Anderson (1951) claim that a method which they have devised employing several buffered sucrose solutions of different specific gravities, yields nuclei which are optically very similar to those within living cells. Unfortunately, since during the isolation process the nuclei tend to form large clumps, the usefulness of this technique is somewhat limited. Recently Dounce (1952a) described briefly how nuclei may be isolated in 1% gum arabic solution adjusted to pH6 with sodium hydroxide. Finally Falconer, Jenden & Taylor (1953) have devised a method for the isolation of nuclei from rat liver using very concentrated solutions of ammonium sulphate. Since this technique results in precipitation of the tissue proteins <u>in situ</u> it would seem particularly suitable for studies of the proteins of the nucleus.

A certain amount of information on the chemistry of the nucleus has also been obtained from the tissue fractionation studies of Schneider, Hogeboom and others (see Schneider & Hogeboom, 1951, for references). The technique used in

such studies is to homogenize the tissue in a suitable medium and then by differential centrifugation to separate the homogenate into a number of fractions (usually a nuclear fraction, a large granule or mitochondrial fraction, a small granule or microsome fraction and a non-sedimentable or cell sap fraction). By analysis of these individual fractions it is possible to determine how enzymes, etc., are localized within the cell. The results obtained for the nuclear fraction by this method must, however, be regarded with considerable reserve since this fraction is generally heavily contaminated with mitochondria, whole cells, etc. Recently, Hogeboom, Schneider & Striebich (1952) have described what is, in effect, a modification of the tissue fraction technique by which, they claim, up to 90% of the nuclei of rat liver may be isolated in a high state of purity.

Attempts have also been made to isolate individual components of the nucleus. The most important of these has been the isolation of chromatin threads first described by Claude & Potter (1943) and Mirsky & Pollister (1943). Although the methods employed by these two groups of workers were different - Claude & Potter (1943) used macerated mouse leukemic tissue as their starting material whereas Mirsky & Pollister (1943) subjected fish erythrocytes to homogenization in a Waring blendor - and their products also

differed in some respects - Claude & Potter's chromatin threads were extended while Mirsky & Pollister's were contracted - both groups agreed in claiming that they had isolated interphase chromosomes (see Mirsky & Ris, 1947a, b; Ris & Mirsky, 1949). This has been contested by Lamb (1949, 1950) who is of the opinion that the structures isolated were no more than fragments of nuclei which had been stretched and torn to pieces during homogenization. This criticism has not been accepted by Ris (1951) who claims that nuclear fragments of this description can be readily distinguished from "isolated chromosomes" by, for example, the presence of a nuclear membrane. The arguments in favour of identifying isolated chromatin threads as interphase chromosomes have recently been summarised by Ris (1951) and Mirsky (1951) as follows:

1). They have the characteristic shape and longitudinal differentiation of chromosomes.

2). They are distinctly double.

3). Under the influence of "uncoiling agents" such as potassium cyanide they unravel into the gyres of a helix.

4). They bear certain morphological resemblances to metaphase chromosomes.

5). Like chromosomes <u>in situ</u> they give a positive Feulgen reaction.

Similar arguments have been put forward by Polli (1952). Pollister (1952a), on the other hand, is now apparently of the opinion that while some of the isolated chromatin threads are almost certainly true interphase chromosomes this may not be true of them all.

One or two reports have recently appeared on the isolation of other components of the nucleus. Vincent (1952), for example, has isolated the nucleoli of starfish oöcytes in sufficient quantity for chemical analysis and Callan (1952) has shown that oöcyte nuclei of the newt Triturus cristatus carnifex are sufficiently large for free-hand dissection. Mazia & Katsuma (1952) have briefly described a method of isolating the mitotic apparatus of dividing cells in sea urchin eggs. Since these methods can be applied only in a few special cases the results obtained by their use may not be universally valid. The same caution applies, though with somewhat diminished force, to the mechanical technique used by some workers to isolate the giant chromosomes of Drosophila salivary glands (Chu & Pai, 1945; Blumel & Kirby, 1948). The recent claim of Dounce (Dounce, 1952a; Dounce & Litt, 1952) to have isolated nucleoli from liver nuclei by what may perhaps be a method of general applicability is therefore of particular interest.

The various methods described above have been applied almost exclusively to animal tissues. Since Behrens' work on the nuclei of rye germ (Behrens, 1938; Feulgen, Behrens & Mahdihassan, 1937) little interest appears to have been taken in the possibility of isolating nuclei from plant tissues. Recently, however, Brown (1951) has published a method for the isolation of nuclei from onion root tips involving the use of pectinase solution. Unfortunately, it is doubtful, judging from the photograph which accompanies his report, whether his product is sufficiently free of cytoplasmic contamination to be used in chemical studies.

1.5 The proteins of the cell nucleus.

The nature and properties of one of the major chemical components of the cell nucleus, namely DNA, have already been discussed. The study of another major component, the nuclear proteins, dates from Miescher's discovery in 1868 of the organic base present in salmon spermatozoa which he called "protamine". This discovery excited little interest. Indeed, it is said (Kossel, 1928) that in the next twenty years only one brief reference to protamine occurs in the literature. In 1884 Kossel reported the occurrence of a protein rich in nitrogen in the nuclei of avian erythrocytes. Like "protamine" the new protein, which Kossel called "histone", was found in salt-like combination with nucleic

acid. Histone was subsequently found to occur also in thymus (Lilienfeld, 1894). In 1894 Kossel isolated from sturgeon sperm a substance very similar to Miescher's protamine. He accordingly proposed that the name "protamine" should be extended to cover both bases and that the individual protamines should be named, after the species from which they were obtained, "salmine" and "sturine". Three years later Kossel (1899) showed by hydrolysis that sturine contained amino-acids, indicating that protamines had a protein-like structure and were thus related to the histones.

Kossel devoted the rest of his life to the investigation of protamines and histones. The results of his work and that of his associates are reviewed in his last publication, a monograph published posthumously in 1928. He found that protamines were very simple basic proteins built up from only a few species of amino-acids. Almost without exception they had a disproportionately high content of arginine. They were found only in the spermatozoa of certain fish and Kossel was able to report the composition of specimens from no less than seventeen species (of which fifteen were teleosts). The histones, also, were definitely basic in character, but they contained a far greater variety of amino-acids and their arginine content was

markedly lower. They were more widely distributed than the protamines having been identified in sperm heads of certain fish (e.g., Gadidae), in avian erythrocytes, and in calf thymus nuclei.

Twenty-five years of research into protein structure have not brought any dramatic developments in our knowledge of the chemistry of the protamines and histones. The last comprehensive review of the subject (Synge, 1943) was indeed largely devoted to a consideration of the work of Kossel and his associates. Since then, however, the use of modern methods of protein analysis by Block, Hamer and others has led to a somewhat better understanding of the nature of both proteins (Block & Bolling, 1945; Tristram, 1947; Hamer & Woodhouse, 1949; Block, Bolling, Gershon & Sober, 1949; Felix, Fischer, Krekels & Rauen, 1950; Waldschmit-Leitz, Kuhn & Zinnert, 1951; Hamer, 1951; Davidson & Lawrie, 1948; Daly, Mirsky & Ris, 1951; Leaf & Eadie, 1952; Brunish, Fairley & Luck, 1951). In particular, it has been confirmed that histones are distinctly more complex than protamines. It should, however, be emphasized that there may exist basic nuclear proteins intermediate in complexity between histones and protamines (Kossel, 1928). The basic protein of fowl sperm, gallin, which was discovered by Daly, Mirsky & Ris (1951) and

Table 7.

Amino-acid composition of salmine (the protamine of salmon sperm).

	Relative numbers of amino-acid residues	
	(1)	(2)
Arginine	47 ±1	40
Alanine	4	1
Glycine	-	3
Isoleucine	1	1
Proline	6	4
Serine	6	7
Valine	3	2
Total	67 ±1	58
Minimum molecular weight	9000	8000

from (1) Block & Bolling (1945),
and (2) Tristram (1947).

Table 8.

Amino-acid composition of protamines and histones.

(Amino-acid nitrogen as % of total nitrogen.)

	Clupein (herring sperm protamine)		Calf thymus histone		Rat liver histone
	(1)	(2)	(3)	(4)	(4)
Alanine	4.7	1.89	6.0	8.5	10.7
Ammonia	-	-	4.8	9.5	7.2
Arginine	87.1	89.7	30.7	20.1	23.2
Aspartic acid	-	-	3.3	5.4	4.45
Glutamic acid	-	-	2.25	6.9	5.68
Glycine	-	-	5.2	6.2	5.94
Histidine	-	-	4.0	4.2	1.62
Isoleucine	1.0	0.43	12.0	3.7	10.0
Leucine	-	-	3.05	6.1	
Lysine	-	-	10.8	8.5	11.6
Phenylalanine	-	-	1.9	1.17	1.65
Proline	8.2	2.22	2.7	3.0	2.1
Serine	3.4	2.42	3.45	4.7	5.0
Threonine	1.9	0.65	3.1	3.53	5.6
Tyrosine	-	-	1.4	0.5	1.07
Valine	-	1.60	4.9	6.1	3.9

from (1) Block et al. (1949)

(2) Felix et al. (1950)

(3) Hamer (1951)

and (4) Leaf & Eadie (1952).

Table 9.

Analyses of the basic proteins isolated from the nuclei of various tissues of the salmon.

Source	Nitrogen content of sulphate (%)	Arginine nitrogen (as % of total nitrogen)
Mature sperm heads	22.6	87.7
Nuclei from unripe testes	20.6	66.5
Erythrocyte nuclei	15.6	19.0
Liver nuclei	15.75	25.3

from Stedman & Stedman (1947a, b).

Table 10.

Analyses of "main" and "subsidiary" histones from various ox tissues.

	Arginine nitrogen (as % of total nitrogen)	Tyrosine (%)
Thymus		
main histone	29.8	4.37
subsidiary histone	11.5	1.40
Spleen		
main histone	29.3	4.33
subsidiary histone	13.3	1.29
Liver		
main histone	30.4	4.32
subsidiary histone	13.8	1.70

from Stedman & Stedman (1951).

classified by them as a protamine might perhaps be placed in this category. So also might the basic proteins of mollusc and sea urchin sperm described by Hultin & Herne (1949). Some data on the composition of basic nuclear proteins are given in Tables 7 and 8.

Although the three sorts of tissue from which Kossel and his associates had isolated protamine or histone (fish sperm, avian erythrocytes and calf thymus) could hardly have been more diverse Kossel himself does not appear to have thought it probable that all nuclei should contain one or other of these basic proteins. The demonstration that this is, in fact, the case was largely the result of applying to a wide variety of tissues the method for the extraction of histone in combination with DNA which was devised by Mirsky & Pollister (1942). By this means, so-called "nucleohistones" similar to those previously obtained from calf thymus were extracted from mammalian liver, kidney, spleen, brain, pancreas and thymus; from frog, shad, trout, and sea urchin sperm; from liver, spleen and blood cells of the dog-fish; and from wheat germ (Mirsky, 1945; Mirsky & Pollister, 1946). At about the same time, Stedman & Stedman began a thorough investigation of the basic proteins which they obtained by extracting nuclei isolated from various tissues with dilute (0.1N) hydrochloric or sulphuric acid. In the course of this work they found, contrary to

their expectations, that the basic proteins of salmon erythrocyte and liver nuclei were not protamines but histones, although salmon sperm had been the tissue in which Miescher originally discovered protamine (Stedman & Stedman, 1944). Moreover, there appeared to be a difference in composition between the erythrocyte histone and the liver histone which was too great to be attributable to experimental error. A subsequent analysis of the basic protein of immature salmon testes gave figures intermediate between those obtained for sperm heads and for liver nuclei. These results indicated quite clearly that the composition of the basic protein of the nucleus could vary from one tissue to another within a single species (Stedman & Stedman, 1947a, b, see also Table 9). More recently Stedman & Stedman (1951) have been able to fractionate the histones obtained from the nuclei of certain tissues, including cod sperm into "main histones" and "subsidiary histones". Mirsky & Ris (1947b) had previously produced evidence for the occurrence of more than one type of histone in calf thymus but this might have been due to different cell types within the tissue containing different histones. Stedman & Stedman's (1951) demonstration of the same phenomenon in spermatozoa, where the cells are all of the same type, indicates the co-existence of two histones within the same nucleus. Some of Stedman &

Stedman's (1951) analyses of main and subsidiary histones are shown in Table 10.

Incredible as it may seem, it would appear to have been generally believed, up to about ten years ago, that histone (or protamine) and nucleic acid were the only quantitatively important constituents of the cell nucleus. This history of this curious concept has been summarised by Stedman & Stedman (1947a). It seems to have had its origin in deductions drawn from Miescher's data by Schmiedeberg, although some at least of Miescher's analyses of nuclei appear to support exactly the opposite conclusion (Miescher, 1896). The first suggestion that this was an over-simplified view of the situation came from the cytochemical studies of Caspersson (1941) which indicated the presence of a higher protein in the nucleus in addition to histone and DNA. In the following year Mayer & Gulick (1942) reported that nuclei which they had isolated by the Behrens technique contained, in addition to DNA and histone, two higher proteins, acidic rather than basic in nature, one of which appeared to be of the globulin type and to have a high content of sulphur. This announcement appears to have attracted little attention, but shortly afterwards Stedman & Stedman (1943) reported the discovery of a non-histone protein in the nucleus which was characterised by

its tryptophan content and by an iso-electric point between pH 3 and 5. Since it stained strongly with such basic dyes as methylene blue and gentian violet and since with haematoxylin it gave the characteristic blue colour seen in the nuclei of a haematoxylin stained section Stedman & Stedman (1943) concluded not only that it was responsible for the staining reactions of the nucleus but also that it was the most important, if not the sole, constituent of the chromosomes. Accordingly, they named it "chromosomin". On analysis chromosomin was found to have a surprisingly high content of basic amino-acids (arginine, 9.5%; histidine, 5%; lysine, 11%). Its acidic properties were presumed to be due to a correspondingly high content of glutamic and aspartic acids. Although qualitative experiments indicated the presence of chromosomin in all nuclei examined Stedman & Stedman were unable to estimate the amount present by direct analysis. Accordingly they assumed that their dry, lipid-free nuclei contained only three quantitatively important constituents, nucleic acid, histone and chromosomin. Nucleic acid could be estimated by phosphorus determinations and histone by extraction with dilute sulphuric acid and gravimetric determination as histone sulphate; chromosomin could then be calculated by difference (Stedman & Stedman, 1943, 1947a, b, 1951). Some of the results obtained by this method are shown in Table 11. The high content of

Table 11.

Composition of dried, lipid free nuclei.

Origin of nuclei	Histone %	Nucleic acid %	Chromosomin %
Ox spleen	16	24	50
Fowl erythrocytes	24	43	33
Calf thymus	21	44	35
Cod sperm	12	28	60
Walker rat carcinoma	1.6	26	72.4
Mouse carcinoma	3	32	65
Chick embryos	3	35	62

from Stedman & Stedman (1943b).

Table 12.

Composition of isolated chromatin threads ("isolated chromosomes").

Source of chromatin threads	Deoxyribonucleic acid (DNA) %	"Residual protein" %
Carp erythrocytes	41	4
Calf thymus	39	8.5
Calf liver	26	39
Calf kidney	28	33
Beef pancreas	28	29

from Mirsky & Ris (1949).

chromosomin (relative to DNA and histone) in all the preparations of nuclei analysed is of particular interest.

A nuclear, non-histone, tryptophan-containing protein has also been obtained by Mirsky and his associates from isolated nuclei and from whole tissue homogenates from which cytoplasmic material has been removed by washing with physiological saline (Mirsky & Pollister, 1946; Pollister & Mirsky, 1946). Alternatively this so-called residual protein may be obtained by treating "isolated chromosomes" with neutral M sodium chloride which removes most of the DNA and histone leaving only the so-called "residual chromosomes"; and treating these in turn with hot dilute trichloracetic acid or physiological saline to remove the remaining nucleic acid. Residual protein may be distinguished from histone by its lower nitrogen content (13% as against 18%), its tryptophan content (1.36% as against 0.14% for thymus histone) and its insolubility (Mirsky & Ris, 1947b). The composition, in terms of DNA and residual protein, of "isolated chromosomes" is shown in Table 12. A protein similar to residual protein has also been obtained by Jeener (1946, 1947). It must be borne in mind that chromosomin and residual protein are not highly characterised individual proteins but rather vaguely defined protein fractions which, in absence of evidence to the contrary, may

be presumed to be more or less heterogeneous. It is not yet clear to what extent "chromosomin" and "residual protein" may be regarded as different names for the same chemical entity.

Since the original observations of Stedman & Stedman (1943) and Mirsky (Mirsky & Pollister, 1946; Pollister & Mirsky, 1946) were published a number of papers have appeared describing nuclear non-histone proteins. Thomas & Mayer (1949) have reported the occurrence in boar sperm of a protein soluble in alkali and precipitated at pH 6. Similar proteins have subsequently been obtained from isolated rat liver nuclei and calf thymus chromosomes (Wang, Kirkham, Dallam, Mayer & Thomas, 1949). More recently, the same group of workers have identified the alkali-soluble protein of isolated rat liver nuclei as a lipoprotein containing phospholipid and cholesterol. They appear to regard it as essentially identical with both chromosomin and residual protein but they suggest that it may be derived from the nuclear membrane rather than the chromosomes. It accounts for nearly 50% of the dry weight of nuclei isolated at pH 6 by the method of Dounce (1943a). Similar proteins have been obtained from the nuclei of calf liver, calf thymus, ox spleen and fowl erythrocytes (Wang, Mayer & Thomas, 1953). Brown, Callan & Leaf (1950) have analysed chromatographically

the proteins in nuclear sap isolated from oöcytes of the newt Triturus cristatus. They conclude from their results that in these cells the nuclear sap contains higher proteins rather than histone or protamine. They emphasize, however, that it would be unwise to draw any general conclusions from experiments on a cell type as extraordinary in its morphology as the amphibian oöcyte. Yasuzumi & Miyao (1950, 1951a, b) have made an extensive chromatographic study of the amino-acid composition of chromosomes isolated from erythrocytes and leucocytes of a wide variety of vertebrate species. Their results confirm the presence of higher protein in the chromosomes and indicate the existence of differences in composition of chromosome protein between different species but not between the leucocytes and erythrocytes of the same species. These conclusions are consistent also with the results obtained by Blumel & Kirby (1948) for the composition of mechanically isolated Drosophila salivary gland chromosomes. The existence of a higher protein in the nucleus would also appear to be confirmed by the amino-acid analyses of hydrolysates of fowl erythrocyte nuclei carried out by Melampy (1948). Unfortunately the significance of the results obtained in this investigation is diminished by the fact that an appreciable proportion of the nuclei analyzed were contaminated with residual stroma.

The incompleteness of present knowledge of the proteins of the nucleus is underlined by the recent discovery by Kirkham & Lloyd (1953) of a third protein in nuclei isolated by the Behrens technique from calf thymus and calf liver. The new protein is quite distinct both from histone and from the nuclear lipoprotein previously described by this group (Wang et al., 1953) and is apparently of the globulin type. It accounts for about 20 - 40% of the dry mass of the nucleus.

1.6 The enzyme activities of the cell nucleus.

Present knowledge of the enzymes of the nucleus is chiefly derived from three sources:

(i) examination of the nuclear fraction obtained in the course of tissue fractionation;

(ii) examination of nuclei isolated in aqueous media;

(iii) examination of nuclei isolated in non-aqueous media.

The results obtained from (i) may be disposed of fairly briefly. It has already been pointed out that nuclear fractions are invariably contaminated to a greater or lesser degree with whole cells, mitochondria, etc. It is therefore unsafe to assume that any enzyme activity found in this fraction is necessarily due to the nuclei unless it accounts for a disproportionately high percentage of the total activity of the tissue. For example, Schneider & Hogeboom (1951) have emphasized that only about

5% of the total cytochrome oxidase activity of the liver is found in the nuclear fraction compared with 75 - 80% in the mitochondrial fraction (Schneider, 1946a ; Schneider & Hogeboom, 1950). Under these circumstances it is impossible to be certain how much of the activity of the nuclear fraction is due to the nuclei themselves and how much to contaminating mitochondria. On the other hand, the nuclear fractions of rat liver (Novikoff, Podber & Ryan, 1950; Ludewig & Chanutin, 1950; Novikoff, Hecht, Podber & Ryan, 1952; Schneider, 1946a), rat hepatoma (Schneider, 1946a), mouse liver and mouse hepatoma (Schneider, Hogeboom & Ross, 1950) account for such a considerable proportion of the adenosine monophosphatase and adenosine triphosphatase activities of these tissues that the nuclei must be presumed to have a high concentration of these enzymes. One other positive result of great interest has been obtained in the course of these studies: it has been shown that, in mouse liver, the mechanism for the synthesis of diphosphopyridine nucleotide is apparently confined to the nucleus (Hogeboom & Schneider, 1952). This observation is of particular significance since the synthesis of diphosphopyridine nucleotide is carried out by a water-soluble enzyme which can be readily extracted from liver (Kornberg, 1950). It appears, therefore, that even in the aqueous isolation medium used by these workers (0.25M sucrose

containing a trace of calcium chloride) the nuclear membrane is capable of retaining a soluble protein.

A fair number of isolated observations have been published on the occurrence of various enzymes in nuclei isolated from various tissues by a variety of methods. Miller & Kozloff (1947) have found ribonuclease in fowl erythrocyte nuclei. Lang, Seibert, Baldus & Corbet (1950) have observed that in pig kidney the concentration of deoxyribonuclease is higher in the nucleus than in the cytoplasm. The occurrence of nucleases in the nucleus has also been reported by Euler, Fischer, Hasselquist & Jaarma (1945) and by Brown & Laskowski (1951). Phosphatase activity has been demonstrated in fowl erythrocyte nuclei (Dounce & Seibel, 1943) and in "residual chromosomes" (Jeener, 1946; Mirsky, 1947). On the other hand, the L-amino-acid oxidase, D-amino-acid oxidase, L-proline oxidase and xanthine oxidase activities of pig kidney nuclei are negligible (Lang & Seibert, 1950) as also is the succinic dehydrogenase activity of rat liver nuclei (Euler et al., 1945). Clearly, such a mass of disconnected data cannot do more than indicate that the nucleus may be capable of catalysing a fairly wide range of reactions.

Fortunately, a lengthy and systematic investigation of the enzymes of the rat liver nucleus was undertaken by

Table 13.

Enzymes of nuclei isolated from rat liver.

Enzyme	Relative Activity* in nucleus
Aldolase	40
D-amino-acid oxidase	100
Arginase	113
Catalase	50 - 60
Choline oxidase	0
Cytochrome oxidase	50 - 60
Cytochrome c	low
Enolase	50
Esterase	50
Lactic dehydrogenase	40
Acid phosphatase	25 - 50
Alkaline phosphatase	192
Phosphorylase	26
Succinic dehydrogenase	0
Uricase	100

* $\frac{\text{Activity per mg. dry wt. of nuclei}}{\text{Activity per mg. dry wt. of whole tissue}} \times 100$

from Dounce (1950a).

Dounce in 1943. The results obtained are shown in Table 13 (see Dounce, 1950a, b). They indicate the presence in the nucleus of a wide range of enzymes, including some of those concerned in the process of glycolysis (aldolase, enolase, lactic dehydrogenase and phosphorylase). On the other hand, the absence of succinic dehydrogenase is notable as indicating that the tricarboxylic acid cycle does not operate within the nucleus. With the exception of alkaline phosphatase most of the enzymes studied were present in the nucleus only in low concentration. For this reason, Schneider and his associates (Schneider & Hogeboom, 1951; Hogeboom, Schneider & Striebich, 1952; see also Schneider, 1946a; Hogeboom & Schneider, 1950; Hogeboom, 1951), have suggested that in these cases Dounce was, in fact measuring the enzyme activity of traces of cytoplasmic contaminants. This implies, of course, that the nucleus, apart from its phosphatase activity and its capacity for synthesizing diphosphopyridine nucleotide, is metabolically rather inert. Dounce (1950a; 1951) does not accept the validity of these criticisms and continues to regard the nucleus as metabolically active. This concept finds a certain measure of support in the fact that avian erythrocyte nuclei, which can be isolated much more easily than the nuclei of compact tissues, have been repeatedly shown to have a slight but quite measurable oxygen consumption and to be capable of

anaerobic glycolysis (Negelein, 1925; Laskowski, 1942; Hunter & Baufield, 1944).

Much more impressive evidence in favour of the "metabolically active nucleus" has come from the work of Stern, Mirsky, Allfrey & Saetren (1952). These workers undertook a large-scale investigation of the enzyme activities of nuclei isolated from a wide variety of tissues by Allfrey's (1952) modification of the Behrens technique. Behrens himself had shown that in the livers of guinea pigs and rabbits the nuclei contained nearly as high a concentration of arginase as the cytoplasm whereas their lipase concentration was comparatively low (Behrens, 1939). Later workers formed the impression that the preliminary freeze-drying required in the Behrens technique would probably inactivate at least some enzymes (Schneider & Hogeboom, 1951). Stern et al. (1952) found, however, that this was a less serious difficulty than had been anticipated since those enzymes which could be prepared as acetone-extracted powders survived their isolation procedure. The enzymes which they studied fell into two classes.

(1). Special enzymes characteristic of a particular tissue, e.g., pancreatic amylase and intestinal phosphatase. The proteins haemoglobin and myoglobin, the distribution of which was also studied, might be

regarded as belonging to this class.

(2). Enzymes of general distribution, such as esterase and β-glucuronidase.

The results of this monumental investigation may be summarised briefly as follows.

(1) The nuclei of different tissues have different properties. For example, calf liver nuclei contain catalase while calf kidney nuclei do not, although the enzyme occurs in both tissues; arginase is found in fowl liver nuclei but not in fowl kidney nuclei; and haemoglobin occurs in erythrocyte nuclei while myoglobin appears to be absent from muscle nuclei.

(2) For enzymes of general distribution the variations in activity between the nuclei of different tissues are as great as, if not greater than, those between the tissues themselves.

(3) Fasting a horse for twenty days causes a catastrophic drop in the catalase activity of both the nuclei and cytoplasm of the liver. The effect on other liver enzymes is much less marked and takes the form of a fall in nuclear activity accompanied by a rise in cytoplasmic activity.

One aspect of the problem which was not covered by the experiments of Stern et al. (1952) is the question of whether the nucleus contains any mechanism for obtaining energy.

Stern & Mirsky (1952) have recently examined the nuclei of wheat germ (isolated by the method of Allfrey et al., 1952) from this point of view. Their results indicate that, in this tissue at least, the nuclei possess a powerful glycolytic mechanism. This, of course, is in agreement with the results of Dounce described above. An alternative theory has been put forward by Lang & Seibert (1951). These workers have found that in the nuclei of liver and kidney isolated in sucrose solution, lactic acid production is slow and adenosine triphosphatase activity is relatively high. They are of the opinion, therefore, that the chief source of energy in the nucleus is not glycolysis but the breakdown of adenosine triphosphate derived presumably from the cytoplasm. The third possibility, that the nucleus might derive some energy from oxidative metabolism, is not supported by the work of either Dounce or Stern. It cannot, however, be entirely ignored in view of the reports, cited above, that the isolated avian erythrocyte nucleus has a measurable respiration and of Zittle & Zitin's (1942a) demonstration of cylochrome oxidase in the isolated heads of bull sperm.

1.7 Other constituents of the cell nucleus.

While DNA, histone and the non-histone proteins are quantitatively the most important constituents of the nucleus

the presence of a number of other components has also been demonstrated. RNA, the occurrence of which was first suspected on histochemical grounds, has been repeatedly found in isolated nuclei (Brachet, 1940a; Davidson & Waymouth, 1944; Bergstrand, Eliasson, Hammarsten, Norberg, Reichard & von Ubisch, 1948; Vendrely & Vendrely, 1948; Dounce et al., 1949; Villela, 1949; Barnum et al., 1950; Marshak, 1951; Allfrey et al., 1952; McIndoe & Davidson, 1952; Mauritzen, Roy & Stedman, 1952). RNA has also been found in isolated chromosomes (Mirsky & Ris, 1947; Petermann & Mason, 1948). The differences in composition between nuclear and cytoplasmic RNA have already been discussed (see Section 1.2 above).

The mineral content of nuclei isolated by the Behrens technique has been studied by Gulick (Williamson & Gulick, 1942; Gulick, 1946). The results which he has obtained are shown in Table 14. The high calcium content is of interest particularly in connection with Barton's (1951) observation that, as the DNA of the nucleus is hydrolysed by deoxyribonuclease, calcium is liberated in stoichiometric amounts. Dounce & Beyer (1948) have estimated the mineral content of nuclei isolated from rat liver in aqueous citric acid at pH 6. Unfortunately, since this isolation procedure must be expected to wash out any free metallic ions from the

Table 14.

Calcium and magnesium content of nuclei isolated by the method of Behrens (1932).

	g. per 100 g. dry weight of nuclei	
	Calcium	Magnesium
Calf thymus nuclei	1.3 - 1.4	0.07 - 0.1
Human tonsil nuclei	1.25	0.07
Nuclei from bovine supermammary lymph node	1.41	0.06

from Williamson & Gulick (1942) and Gulick (1946).

Table 15.

Metals present in nuclei isolated at pH 6 by the method of Dounce (1943a).

Element	mg. per 100 g. dry weight of nuclei.
Cu	25
Al	17
Fe	11
Mg	7
Mn	1.4
Zn	1.2
Ca	0.23

from Dounce & Beyer (1948).

nuclei the interpretation of their results (shown in Table 15) is a matter of some difficulty.

The occurrence of lipids, including cholesterol and phospholipids, in the cell nucleus was first established for muscle and tumour nuclei by Stoneburg (1939). Lipids have subsequently been demonstrated in nuclei isolated from rat carcinosarcoma (Haven & Levy, 1942), rat liver (Dounce, 1943b), fowl erythrocytes (Dounce & Lan, 1943), snake erythrocytes (Villela, 1947) and human cerebral cortex (Tyrrel & Richter, 1951). The most systematic investigation of the nuclear lipids yet undertaken is that carried out by Williams, Kaucher, Richards, Moyer & Sharpless (1945). These authors found that nuclei isolated in aqueous citric acid at pH 6 from normal dog liver, normal rat liver and cancerous rat liver all contained about 4 - 5% by weight of neutral fat, 2 - 3% of cephalin, 4 - 9% of lecithin, about 0.5% of sphingomyelin, about 1% of cholesterol esters, and smaller variable amounts of cerebroside and free cholesterol. In each case the total lipid content was 14 - 18%, of which roughly two-thirds was phospholipid.

The presence of certain of the B vitamins in the nucleus was demonstrated by Isbell, Mitchell, Taylor & Williams (1942) who found thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, biotin, inositol and

folic acid in nuclei isolated by the Behrens technique from beef heart muscle and from a mouse tumour. The concentrations of these vitamins in the nuclei were comparable with those found in the corresponding whole tissue. Villela (1947) has also found measurable concentrations of thiamine and pyridoxine in isolated snake erythrocyte nuclei.

1.8 Cytochemical methods.

While a great deal of valuable information has been obtained by analysis of isolated nuclei this method can obviously give no indication of the localization of the nuclear components within the nucleus. To some extent this deficiency may be remedied by analysis of isolated chromatin threads but it must always be remembered (a) that the identification of these as isolated interphase chromosomes is still a matter of controversy (Lamb, 1949, 1950; Ris & Mirsky, 1949, 1951; Ris, 1951; Mirsky, 1951; Polli, 1952) and (b) assuming that they are isolated chromosomes, the degree to which they are contaminated with non-chromosomal material (e.g., nucleoli and shreds of nuclear membrane) has not yet been clearly established. Fortunately, methods have been devised whereby proteins and nucleic acids may be identified in a histological tissue section either by their absorption of ultraviolet light or by their staining reactions.

The first microscope with quartz lenses suitable for use in the ultraviolet was that designed by Kohler (1904). This instrument was, however, valued chiefly because the shorter wavelength of ultraviolet radiation gave it a higher resolving power than ordinary microscopes working in visible light, and because the various structures of the cell could be distinguished by the different degrees to which they absorbed ultraviolet, thus obviating the necessity for staining and the attendant danger of staining artefacts. Kohler (1904) used it to photograph chromosomes, and many years later Lucas & Stark (1931) and Wyckoff, Ebeling & Ter Louw (1932) employed it in studies of mitosis, the latter group of workers remarking on the similarity between photomicrographs of unstained mitotic figures taken at a wavelength of 275 mμ. and photographs in visible light of the same objects after Feulgen staining. It was left, however, to Caspersson (1936) to demonstrate that it was possible to identify proteins and nucleic acids in tissue sections by their characteristic ultraviolet absorption spectra and that at 260 mμ. the absorption of the nucleic acids was so great as to completely dominate that of the proteins. (It is a curious fact that the very high ultraviolet absorption of the nucleic acids had been observed in vitro by Dhéré in 1906 only two years after Köhler's description of his quartz microscope. This observation

seems to have been completely forgotten until 1931 when it was confirmed by Heyroth & Loofbourow.)

It would be quite impossible to attempt to summarise here Caspersson's monumental work on the relations between nucleic acid and protein, particularly as it was directed towards RNA and the cytoplasm rather than DNA and the nucleus (Caspersson, 1947, 1950; Caspersson & Schultz, 1939, 1940; Caspersson, Nystrom & Santesson, 1941; Caspersson, Landstrom & Aquilonius, 1941; Hyden, 1943, 1947). Some of his results are, however, highly relevant to any study of the cell nucleus. They are as follows:

(1) The ultraviolet absorption spectrum of a metaphase chromosome is very similar to that of purified nucleic acid. The same is true of the dark bands in the giant interphase chromosomes of Drosophila salivary glands (Caspersson, 1936). These observations, which imply that the nucleic acid of the nucleus is localised on the chromosomes have been repeatedly confirmed (Caspersson, 1939, 1940, 1950; Caspersson & Schultz, 1938).

(2) The nucleoli also have an absorption spectrum indicative of the presence of nucleic acid but since, unlike the chromosomes, they do not give a positive Feulgen reaction they are thought to contain RNA

rather than DNA (Caspersson & Schultz, 1940; Caspersson, 1941, 1950).

(3) The absorption spectra of nucleoli and chromosomes indicate that both contain proteins as well as nucleic acids (Caspersson, 1941, 1947, 1950).

The apparatus used by Caspersson and his associates to obtain these results is extremely complex and expensive (Caspersson, 1936, 1940, 1947, 1950; Thorell, 1947; Caspersson & Schultz, 1951). Fortunately, it has been possible to confirm and extend many of their findings by simpler methods. Brachet (1940a, b, 1942) has shown that after a tissue section is treated with a solution of ribonuclease (which is known to catalyse the hydrolysis of purified RNA) the nucleoli no longer stain with pyronin, thus confirming that they contain RNA. In agreement with this observation, Davidson & Waymouth (1946) found that ribonuclease treatment also decreased the ultraviolet absorption of the nucleolus. The occurrence of both DNA and protein in Drosophila salivary gland chromosomes was confirmed when Mazia & Jaeger (1939) showed that treatment with a crude nuclease preparation reduced their Feulgen stainability while leaving a residue which stained with ninhydrin. Caspersson (1936) had previously shown that treatment with trypsin caused complete disintegration of

the chromosomes. Pepsin, on the other hand, caused marked shrinkage but no loss of continuity or reduction of Feulgen stainability (Frolova, 1944; Mazia, Hayashi & Yudowitch, 1947). These observations have since been confirmed by Kaufman, Gay & McDonald (1950). Since Mazia et al. (1947) find that pepsin, unlike trypsin, does not digest histone or nucleohistone in vitro they suggest that the shrinkage caused by pepsin treatment is due to removal of the non-histone protein from the chromosome, leaving histone, which maintains the continuity of the fibre. Destruction of this continuity and consequent disintegration of the chromosome are produced by trypsin, presumably because it removes both proteins. It has also been suggested that the chromosomes may contain RNA, the evidence for this being that their staining reactions change slightly after treatment with ribonuclease (Brachet, 1940b, 1942; Kaufman, 1949; Kaufman, McDonald & Gay, 1948; Kaufman, Gay & McDonald, 1950, 1951; see also Serra, 1947).

No discussion of cytochemical methods would be complete without some reference to the method devised by Gomori (1939) for the localization of phosphatase activity in tissue sections. The usual procedure is to incubate the section in a solution containing substrate (usually glycerophosphate) and calcium ions. Phosphate ions liberated from the substrate at the site of enzyme activity

are theoretically precipitated *in situ* as calcium phosphate. By addition of cobalt nitrate the precipitate is converted to cobalt phosphate which is in turn converted to cobalt sulphide, which is in an intensely black substance and may easily be observed microscopically. Using this method Willmer (1942) and Wachstein (1945) reported that phosphatase was present in high concentration in the chromosomes. Danielli & Catcheside (1945) went further and claimed that in the salivary gland chromosomes of *Drosophila* phosphatase activity was localized in the Feulgen-positive bands. Further interest was aroused when in 1948 Brachet & Jeener suggested, on the basis of similar experiments, that phosphatase activity might be closely related to DNA metabolism (see also Brachet, 1947). Unfortunately, it was shown shortly afterwards that, as a method of locating phosphatase activity *within the cell*, the Gomori technique may be highly misleading (Martin & Jacoby, 1949; Pallade, 1951; Novikoff, 1951, 1952; Gomori, 1951). Apparently, the calcium phosphate formed in the course of the test is fairly diffusible and is selectively adsorbed by the chromosomes. The whole episode is a striking illustration of the dangers of placing too much reliance on cytochemical results unsupported by confirmatory evidence from other sources.

1.9 Isotope experiments on nucleic acids.

The investigations described above have yielded a considerable amount of information about the chemical nature of the nucleus. The picture which they present, however, is essentially static. Even the enzyme experiments indicate only what reactions may take place in the nucleus, not what reactions do take place or into what systems these reactions are organised. It is, of course, possible to interpret features of this static picture as evidence for the existence of some dynamic process. Caspersson (1947, 1950) has argued from the occurrence of gradients in the concentration of protein from the nucleolus to the nuclear membrane, and in the concentration of nucleotides from the nuclear membrane outwards into the cytoplasm that protein synthesized by the nucleolus-associated chromatin diffuses from the nucleolus to the nuclear membrane, on the outside of which ribose nucleotides (including presumably RNA) are produced, and in turn diffuse out into the cytoplasm (Caspersson, 1939, 1941; Hyden, 1943). In a somewhat similar fashion Stedman & Stedman (1943b) have suggested that since nuclei of tumour and embryonic tissue have a much lower content of histone than thymus and erythrocyte nuclei histones probably act as inhibitors of mitosis. Obviously, in the absence of supporting evidence theories such as these must be regarded with considerable reserve.

The most important experiments on the actual metabolism of the nucleus have been isotopic studies on the rates of synthesis and breakdown of the nucleic acids in animal tissues. In the earliest of these investigations it was shown that the incorporation of radioactive phosphorus into DNA was generally very slow (Hahn & Hevesy, 1940; Hevesy & Ottesen, 1943; Ahlstrom, Euler & Hevesy, 1945). The most active incorporation of phosphorus into DNA was found in intestinal mucosa, a tissue in which it is well known that the individual cells have only a short life-span and are continually being replaced (Hevesy & Ottesen, 1943). Incorporation was also higher in the tissues of embryos and young, growing animals, in which mitoses are presumably frequent, than in adult tissues, in which presumably they are rare (Ahlstrom, Euler & Hevesy, 1944; Andreassen & Ottesen, 1945; Davidson, 1947). Following partial hepatectomy in the rat the remaining fragment of liver undergoes a remarkably rapid hyperplasia; while this continues the incorporation of phosphorus into the liver DNA is increased markedly above the normal resting level. The DNA phosphorus turnover in hepatoma, another rapidly growing tissue, is also higher than in normal liver (Brues, Tracy & Cohn, 1944). Irradiation with X-rays, a treatment known to have an injurious effect on the chromosomes (Lea, 1946), decreases the incorporation

of phosphorus into DNA (Ahlstrom, Euler & Hevesy, 1945; Hevesy, 1948).

Somewhat similar results were obtained using the heavy isotope of nitrogen. Davidson & Raymond (1947) found only slight incorporation of isotopically labelled ammonia into the DNA of pigeon liver. In the rat, labelled adenine is incorporated more slowly into the purines of DNA than into those of RNA (Brown, Petermann & Furst, 1948). Incorporation of labelled glycine into the purines of DNA is more rapid in liver regenerating after partial hepatectomy than in normal liver (Bergstrand et al., 1948). It seems reasonable, therefore, to suppose that the rate of synthesis of DNA in a resting tissue is extremely low but that it is increased in those tissues in which mitoses are frequent. This is, of course, quite consistent with its localization in the chromosomes.

In general the RNA of normal animal tissues which are not growing rapidly is metabolically much more active than the DNA in respect of turnover of both phosphorus (Brues, Tracy & Cohn, 1944; Hammarsten & Hevesy, 1946; Davidson, 1947; Reichard, 1949) and nitrogen (Davidson, 1947; Brown, Petermann & Furst, 1948; Bergstrand et al., 1948; Reichard, 1949). The RNA of the nucleus appears to have a more rapid turnover than the RNA of the cytoplasm

(Bergstrand et al., 1948; Marshak & Calvet, 1949; Barnum & Huseby, 1950; Jeener & Szafarz, 1950; McIndoe & Davidson, 1952).

1.10 The bacterial transforming factors.

Although the nucleic acids were discovered more than eighty years ago and although their presence has since been demonstrated in all types of plant and animal cells investigated (Davidson, 1947, 1950), in bacteria (Vendrely & Lehoult, 1946; Stacey, 1947; Belozersky, 1947; Chargaff, 1947; Malmgren & Heden, 1947; Vendrely, 1950), and in viruses (Bawden & Pirie, 1937, 1938; Stanley, 1940; Knight, 1947) it is only within the last ten years and in a limited number of cases that it has been possible to show that a purified nucleic acid may have a biological activity in the same sense as purified pepsin or purified insulin. The manner in which this biological activity was discovered is of some interest.

One of the most considerable achievements of the science of immunology has been the classification of the pneumococci into a large number of different "types" each characterised by the possession of a specific, serologically distinct, capsular polysaccharide (Boyd, 1947). In 1928 Griffith described how non-encapsulated, non-virulent pneumococci, characterised by the rough appearance of their

colonies on solid media and called therefore "R" forms, could be obtained from the capsulated, virulent, smooth colony, or "S" strains by culturing on chocolate blood agar or in the homologous immune serum. This change was accompanied by loss of the serological type character. If a living culture of an R strain was inoculated into mice together with a killed culture of the S strain of the same type the living pneumococci which were recovered from the bodies of mice which succumbed to pneumonia were of the S strain. This R ⟶ S conversion could also be produced using a killed S culture of a different type but in this case the living organisms recovered belonged, not to the type from which the R strain had been originally derived, but to the same type as the killed S culture. For example the R strain (RI) derived from the smooth type I (SI) strain would revert to SI if it was inoculated with a killed SI culture; if, on the other hand, it was inoculated with a killed SII culture it would be converted to SII. In this way any type of pneumococcus could be "transformed" into any other type by inoculating the R variant of the first type and the killed S variant of the second type simultaneously into a mouse.

Griffith's observations were confirmed by Dawson (1930) who also showed that the organisms produced as a result of such a transformation possessed all the

characteristics of normal organisms of the same type and that, when subcultured, they maintained this character without any tendency to revert to the type from which they were originally derived. In 1931 Dawson & Sia succeeded for the first time in producing transformation in vitro by growing R cells in a fluid medium containing the homologous antiserum and in presence of heat-killed S cells. As in Griffith's in vivo experiments, the growing R culture was transformed to the S type of the killed cells. In the same year Dawson & Warbasse (1931) reported direct transformation of type SII to type SIII, using a killed culture of SIII but without going through the intermediate R form. A further advance was made when Alloway (1932) eliminated the heat-killed S cells and produced specific transformation of R cultures using crude cell-free extracts of the appropriate S strain. In a later paper Alloway (1933) reported that the same results could be obtained using a sodium deoxycholate extract of S cells purified by treatment with animal charcoal and capable of passing through a Berkefeld filter without appreciable loss of activity. The nature of the active principle in Alloway's extracts remained obscure for more than ten years, although the work of Sia & Dawson (1931) indicated that it was not the type-specific capsular polysaccharide itself. In 1944 Avery,

Macleod & McCarty obtained from type SIII pneumococci a preparation capable of transforming an R variant derived from SII to SIII. This material was prepared by extracting heat-killed SIII cells with sodium deoxycholate solution, precipitating the active principle with alcohol, redissolving it in saline and deproteinising by the method of Sevag, Lackman & Smolens (1938). Finally, any capsular polysaccharide present was removed by treatment with a bacterial enzyme and the active principle was further purified by alcohol precipitation. The purified material gave a colourless viscous solution in saline. The biuret and Millon reactions on both the dry material and concentrated solutions were negative. The diphenylamine reaction for DNA (Dische, 1930) was strongly positive. The orcinol test, which is given strongly by RNA and weakly by DNA (Schneider, 1946c), was weakly positive. Repeated extraction with alcohol and ether caused no loss of activity. Elementary analyses of four preparations of the dried material were in remarkably good agreement with the theory for the sodium salt of DNA. The transforming activity was not reduced by treatment with ribonuclease or with trypsin and chymotrypsin singly or in combination. The effects of a number of crude enzyme preparations from various sources were also tested. The transforming activity was abolished by those preparations capable of depolymerising purified

DNA *in vitro* and only by such preparations. On examination in the analytical ultracentrifuge the purified material gave a single sharp boundary indicating that it was homogeneous. The transforming activity sedimented at the same rate as the optically observed boundary. Similarly, electrophoresis showed a single fast-moving boundary with which the transforming activity migrated. Transformation could be induced by very small amounts of the purified material (0.02 - 0.003 ug). Similar preparations could be obtained from other pneumococcal types (McCarty & Avery, 1946). Subsequently a purified deoxyribonuclease was obtained from beef pancreas (McCarty, 1946) and it was shown that this enzyme was capable in minute amounts of irreversibly inactivating the transforming factor (McCarty & Avery, 1946).

In the face of this mass of evidence it seems impossible to avoid the conclusion that the pneumococcal transforming factors are highly polymerised DNAs.

The induction of similar bacterial transformations has been reported in the case of *Shigella paradysenteriae* (Weil & Binder, 1947), *Bacillus anthracis* and *Bacillus mesentericus* (Manniger & Nogradi, 1948) and *Escherichia coli* and *Proteus OX 19* (Dianzani, 1950). Boivin and his associates have produced transformations in *E. coli* using

transforming factors which are resistant to the action of pepsin and ribonuclease but are inactivated by deoxyribonuclease and are therefore presumably, like the pneumococcal transforming factors, highly polymerized DNAs (Boivin, Vendrely & Lehoult, 1945a, b; Boivin, Delaunay, Vendrely & Lehoult, 1945a, b, c, 1946; Boivin, 1947). It seems therefore that a wide range of bacterial species may be capable of undergoing transformation reactions.

The nature of the transformation reaction itself or, as it has sometimes been called, the directed mutation has also been the subject of investigation. In particular, it has been shown that in the pneumococci the transformation R $\longrightarrow$ S is a stepwise process (MacLeod & Krauss, 1947; Taylor, 1949a, b; see also Dawson, 1934) and that transforming factors can be obtained from R variants which will induce the reverse transformation S $\longrightarrow$ R. An even more significant development has been the discovery that the pneumococci possess somatic protein antigens (Austrian & MacLeod, 1949a) and that these also may undergo directed mutation under the action of transforming factors quite independently of the capsular polysaccharides (Austrian & MacLeod, 1949b).

1.11 The DNA content of the cell nucleus.

The identification of the transforming factors as highly polymerized DNAs, if it is correct, is a discovery of major importance. Since bacterial transformation reactions are highly specific (type III transforming factor, for example, will induce mutation to SIII and not to SI, SII, SIV, etc.) it follows that the DNAs which induce them must have an equally high degree of specificity. Moreover, in the higher plants and animals DNA is the characteristic chemical component of the nucleus and, as has been shown above (section 1.8), is very probably localized in the chromosomes. Recent work by Robinow, De Lamater and others has indicated that the bacterial cell also has a nucleus with chromosomes (Robinow, 1942, 1945; Tulasne, 1947; Knaysi, 1951; De Lamater, Hunter & Mudd, 1952) and that this nucleus contains DNA (Vendrely & Lehoult, 1946; Tulasne & Vendrely, 1947a, b). Under these circumstances it seems not unreasonable to suppose that, in bacteria at least, the genes may be highly specific macromolecules of DNA and that directed mutations may be due to one or more of these macromolecules derived from a killed donor cell penetrating the nucleus of a living recipient cell (Boivin, 1947; Boivin & Vendrely, 1947).

It might be reasonably expected that in the present state of bacterial cytology it would be no easy matter to

Table 16.

Average deoxyribonucleic acid (DNA) content of nuclei isolated from various tissues.

Tissue	pg* DNA per nucleus
Beef liver	6.4
" thymus	6.6
" kidney	6.0
" pancreas	6.9
Calf liver	6.4
" thymus	6.4
Bull sperm	3.3
Swine liver	5.0
" kidney	5.2
Dog liver	5.0
" kidney	5.3
Mouse liver	6.0
" kidney	5.0
Duck erythrocytes	2.3
" liver	2.1

from Vendrely & Vendrely (1948, 1949c).

* 1 pg. = 10^{-12} g.

Table 17.

A. Average deoxyribonucleic acid (DNA) content of nuclei isolated from the tissues of various species.

Species	pg.* DNA per nucleus		
	erythrocyte nuclei	liver nuclei	sperm
Domestic fowl	2.34	2.39	1.26
Shad	1.97	2.01	0.91
Carp	3.49	3.33	1.64
Brown trout	5.79	-	2.67
Frog	15.0	15.7	-
Toad	7.33	-	3.70
Green turtle	5.27	5.12	-

B. Average DNA content of nuclei isolated from various bovine tissues.

Tissue	pg.* DNA per nucleus
Bull sperm	2.82
Calf thymus	7.15
Calf lymph node	7.03
Calf kidney	6.25
Beef kidney	6.81
Calf liver	6.22
Beef liver	8.4

from Mirsky & Ris (1949).

* 1 pg. = 10^{-12} g.

Table 18.

Average deoxyribonucleic acid (DNA) content of nuclei isolated from various rat tissues.

Tissue	pg.* DNA per nucleus
Liver (normal)	5.9 - 9.4
Liver tumours	6.0 - 8.1
Liver (from tumour-bearing animals)	5.3 - 6.4
Tumour of face	5.9
Thymus	6.1 - 6.3
Spleen	6.0 - 6.5
External orbital gland	21.3

from Cunningham et al. (1950).

* 1 pg. = 10^{-12} g.

Table 19.

Average deoxyribonucleic acid (DNA) content of nuclei isolated from various tissues of the domestic fowl.

Tissue	pg.* DNA phosphorus per nucleus
Erythrocytes	0.249
Liver	0.256
Kidney	0.220
Spleen	0.254
Heart	0.245
Pancreas	0.261
Brain**	0.222
Muscle**	0.242

from Davidson, Leslie, Smellie & Thomson (1950) and Davidson & Leslie (1950b).

* 1 pg. = 10^{-12} g.

** embryonic tissues.

obtain independent confirmatory evidence for this concept. The problem was, however, solved in a most ingenious manner by Boivin, Vendrely & Vendrely (1948). It is a commonplace of cytology that in a given organism all the somatic nuclei (with the exception of those which are polyploid) contain the same number of genes while the gametes, being haploid, contain only half this number. If, in fact, the genes are macromolecules of DNA it follows that all the somatic nuclei of an organism will contain the same amount of DNA and the gamete nuclei will contain half this amount. Accordingly Boivin et al. (1948) isolated very large numbers of nuclei by the citric acid method from calf thymus, liver, pancreas and kidney. The number of nuclei present in each preparation was estimated by counting in a haemocytometer and the total DNA content of the preparation determined by gross chemical analysis. The average DNA content of the nuclei could then be found by simple division. In this way it was found that the average DNA content per nucleus was practically the same for the four tissues investigated and for different individual animals - 6.5×10^{-9} mg. Bull spermatozoa, which were also analysed gave a figure of 3.4×10^{-9} mg. DNA per nucleus, i.e., approximately half the average content of the somatic nuclei. These observations are clearly in excellent agreement with the theory put forward by Boivin

et al. (1948) and which we may conveniently refer to as the Boivin-Vendrely hypothesis. Further evidence of the same sort, some of which is shown in Table 16, was subsequently presented by Vendrely & Vendrely (1948, 1949a, b, 1950).

In 1949 Mirsky & Ris published a series of observations on the average DNA content of nuclei isolated in bulk from animal tissues, and counted and analysed by methods similar to those used by Boivin et al. (1948). In two important respects their results, which are shown in Table 17, differed from those obtained by Boivin and his associates: beef liver nuclei were reported as having a higher average DNA content than the nuclei of other beef and calf tissues; and the average DNA content found for bull spermatozoa was less than half that found for the nuclei of any of the somatic tissues of the same species. In the following year Cunningham, Griffin & Luck (1950) also published figures for the average DNA content of nuclei, isolated this time from rat tissues. These also were not consistent with the Boivin-Vendrely hypothesis, particularly with respect to the high figures obtained for liver and external orbital gland (see Table 18). These early experiments were open to criticism on the ground that the range of tissues investigated in any one species was insufficiently wide. Davidson, Leslie, Smellie & Thomson (1950) accordingly estimated the DNA content per

nucleus for all the tissues of the domestic fowl from which nuclei could readily be isolated. Their results, which are shown in Table 19, suggest strongly that the Boivin-Vendrely hypothesis is valid for this species. On the other hand, further evidence that the hypothesis might not hold for mammalian tissues came from the finding of Klein, Kurnick & Klein (1950) that mouse ascites tumour cells contained two or three times as much DNA as normal mouse liver and kidney nuclei. Another anomaly came to light when Vendrely & Vendrely (1949b) and Schmidt, Hecht & Thannhauser (1949) showed that in Arbacia the average DNA content of the spermatozoa is very much less than that of the ova.

These results were all obtained by analysis of counted suspensions of isolated nuclei. It is, however, possible to estimate directly the relative DNA content of individual nuclei. Mention has already been made of the fact that Caspersson (1936, 1950) has devised an ultraviolet microspectrophotometer suitable for determining the absorption spectrum of a small part of a cell. This instrument may also be used to determine the nucleic acid content of, for example, one of the bands in the salivary gland chromosomes of Drosophila. Since, however, both nucleic acids have the same ultraviolet spectrum it cannot distinguish between DNA and RNA. In 1947 Pollister & Ris described a much simpler apparatus (see also Pollister &

Moses, 1949) whereby the absorption of a small part of a cell at a predetermined wavelength could be measured. They suggested that it might be used to determine the relative amounts of stain in individual nuclei stained by the Feulgen method and hence to estimate the relative amounts of DNA which they originally contained. By this means Mark & Ris (1949) showed that the smallest spherical nuclei in certain rat liver tumours contained the same amount of DNA as similar nuclei in normal rat liver. Other results obtained using the same method gave less support to the Boivin-Vendrely hypothesis. Thus Ris & Mirsky (1949a) found that in rat liver the largest nuclei contained twice as much DNA as the medium-sized nuclei and these in turn contained twice as much as the smallest nuclei. Schrader & Leuchtenberger (1949), on the other hand, found that in the plant Tradescantia paludosa the DNA content of individual nuclei varied widely and quite irregularly from one tissue to another.

Thus by the summer of 1950 the validity of the Boivin-Vendrely hypothesis had become subject to serious doubts. The obvious importance of the hypothesis and particularly its bearing on the question of the chemical nature of the gene made it highly desirable that these doubts should be finally resolved one way or the other. Accordingly, it was

decided to undertake a large-scale investigation into the DNA content of the nuclei in the different tissues of a single mammalian species. The lines on which this investigation was conducted are described in the next section.

Part II.

The Average Deoxyribonucleic Acid Content of the Nuclei of Various Rat Tissues as Determined by Chemical Analysis.

The Average Deoxyribonucleic Acid Content of the Nuclei of Various Rat Tissues as Determined by Chemical Analysis.

2.1 The objects of the investigation.

The only way in which the truth of the Boivin-Vendrely hypothesis could be really convincingly demonstrated for any given species would be by measuring accurately the DNA content of a large number of individual nuclei from a wide variety of different tissues and a large number of different individuals of that species. As has been pointed out above (Section 1.11), a method for estimating the relative DNA content of individual nuclei has been devised by Pollister and his associates (Pollister & Ris, 1947; Ris & Mirsky, 1949a; Pollister & Moses, 1949; Pollister, 1950) and similar "cytophotometric" methods have subsequently been described by other workers (Di Stefano, 1948a, b; Swift, 1950a, b; Pasteels & Lison, 1950a, b, c; Kurnick, 1950; Leuchtenberger, 1950; Naora, 1951; Leuchtenberger, Vendrely & Vendrely, 1951; Leuchtenberger, Leuchtenberger, Vendrely & Vendrely, 1952; Frazer & Davidson, 1953). Unfortunately, these techniques are rather inaccurate and so very laborious as to make them unsuitable for a large scale investigation.

An alternative method of testing the hypothesis is that originally used by Boivin, Vendrely & Vendrely (1948) themselves, namely, to isolate very large numbers of nuclei

from various tissues, to estimate the total number of nuclei in each preparation by counting in a haemocytometer and the total amount of DNA by gross chemical analysis, and then by simple division to calculate the average DNA content per nucleus for each tissue. This chemical method is very much less laborious and time-consuming than any of the cytophotometric methods. It cannot, of course, reveal differences in DNA content between individual nuclei within the preparations analysed, but if it could be shown that all the tissues of a given species had the same average DNA content per nucleus and that this content was not affected by, for example, the age or the nutritional status of the animal, it would be difficult to avoid the conclusion that all the somatic nuclei in that species contained the same amount of DNA.

For these reasons the chemical method was employed in the present series of experiments.

The species chosen for investigation was the rat. There were two reasons for this choice:

(i) Although the rat is one of the commonest laboratory animals comparatively little information had been published on the DNA content of its nuclei.

(ii) Preliminary experiments (Thomson, 1950) indicated that the average DNA content per nucleus might be higher in rat liver than in other rat tissues.

Answers were sought to seven specific questions.

(i) Does the average DNA content per nucleus vary from one tissue to another?

(ii) Does the average DNA content per nucleus in the liver vary with age, sex, strain, or body weight?

(iii) Is the average DNA content per nucleus in the liver altered during pregnancy?

(iv) Is the average DNA content per nucleus in the liver affected

(a) by fasting,

(b) by protein deficiency,

(c) by thiamine deficiency,

(d) by administration of a high-fat diet?

(v) Is the average DNA content per nucleus in the liver affected by administration of thioacetamide in a concentration of 0.032% in the diet? (It has been shown by Rather, 1951, that this treatment causes a dramatic increase in the volume of the liver nuclei.)

(vi) Does diabetes, induced by injection of alloxan, affect the average DNA content per nucleus in the liver?

(vii) Is the average DNA content per nucleus the same in liver tumours as in normal liver?

If any one of these seven questions could be answered in the affirmative the Boivin-Vendrely hypothesis, in its original form at least, would have to be discarded. Conversely,

while negative answers to all seven could not be regarded as a rigorous demonstration of the truth of the hypothesis they would constitute very strong evidence in its favour.

An eighth question was also investigated. It is a well known fact that if the median and left lateral lobes of the liver of a rat are surgically removed the remaining lobes undergo a process of compensatory hyperplasia, commonly but somewhat inaccurately termed regeneration, until the organ has been restored to its original weight (Higgins & Anderson, 1931). During the first four days following the operation the frequency of mitoses in such a regenerating liver is extraordinarily high (Brues, Drury & Brues, 1936; Abercrombie & Harkness, 1951). It seemed of some interest to compare the average DNA content per nucleus under these circumstances with that found in normal liver, in which mitoses are extremely rare (Brues & Marble, 1937; Marshak & Byron, 1945).

Quite apart from its theoretical significance the Boivin-Vendrely hypothesis has a corollary of some practical importance. If all the nuclei in a given species contain the same amount of DNA it follows that the number of cells present in an organ or a sample of tissue can be calculated from its content of DNA, and hence its composition or enzyme activity can be expressed in terms of average composition or

activity per cell (Davidson & Leslie, 1950a). In the course of the present investigation an attempt has been made to determine whether this method of expressing the results of tissue analyses has any considerable advantages over those more commonly employed.

2.2 Experimental Methods.

Animals. Except where otherwise stated the experiments were carried out on male albino rats weighing 190 - 250 g. from the departmental colony. Where indicated in the tables of results, male albino rats outside this weight range, female albino, and male and female hooded rats were also used.

Diets. The diets employed were as follows:

(a) A stock diet of "rat cake" made by Levers Cattlefoods Ltd.

(b) A semi-synthetic diet similar to that used in the University of Illinois consisting of

glucose	73%
vitamin-free casein	18%
salt mixture	4%
arachis oil	5%

Each kg. of diet contained the following supplement:

Thiamine hydrochloride	2.5 mg.
riboflavin	5.0 mg.
nicotinic acid	10.0 mg.
pyridoxin hydrochloride	2.5 mg.
calcium pantothenate	20.0 mg.
inositol	100.0 mg.
p-aminobenzoic acid	50.0 mg.
biotin	0.1 mg.
folic acid	1.0 mg.
2-methylnaphthaquinone	1.0 mg.
choline chloride	1.0 g.

Each animal received one drop weekly of a mixture of 1 g. α-tocopherol in 14 g. Radiostoleum (British Drug Houses Ltd.). The salt mixture employed was that described by Griffin, Nye, Noda & Luck (1948).

(c) A protein-free diet similar to (b) above in which casein was replaced by glucose and additional phosphate was supplied as recommended by Kosterlitz (1947).

(d) A thiamine-deficient diet similar to (b) above but without thiamine hydrochloride.

(e) A thioacetamide-containing diet similar to (b) above but containing in addition 0.032% of thioacetamide.

(f) A high-fat diet as described by Channon, Mills & Platt (1943) containing

casein	8%
beef fat	40%
glucose	46%
salt mixture	5%
cod liver oil	1%

Each rat received 10 µg. thiamine per day.

(g) A carcinogenic diet similar to that used by Griffin et al. (1948) containing

casein	18%
glucose	73%
arachis oil	5%
salt mixture	4%
p-dimethylaminoazobenzene	0.06%

and vitamin supplement. Control animals were kept for the same time on the same diet without the dimethylaminoazobenzene.

Partial hepatectomy. Partial hepatectomy (removal of median and left lateral lobes) was carried out under ether anaesthesia by the method of Higgins & Anderson (1931).

Induction of diabetes. Diabetes was induced by administration of alloxan as described by Diermeier, Di Stefano, Tepperman & Bass (1951). The animals concerned were fasted for 48 hours and then injected subcutaneously with 175 mg. alloxan monohydrate per kg. body weight. Control animals which had been similarly fasted received injections of saline. The development of diabetes was confirmed by blood-sugar estimations by the Folin-Wu method. Animals which did not develop diabetes were discarded.

Removal and storage of tissues. The animals were killed by exsanguination under ether anaesthesia. The livers and other organs required were quickly excised, weighed and finely chopped with scissors. A small piece of tissue was fixed for histological examination, and about 0.5 g. was accurately weighed out for whole tissue analysis. The remainder was used for isolation of nuclei. Tissues which could not be used immediately were preserved by freezing in solid carbon dioxide.

Isolation of nuclei. The methods available for the isolation of nuclei from compact tissues have already been discussed (Section 1.4). For the present investigation the

citric acid method was adopted as being the only one which is applicable to a wide range of tissues and is yet sufficiently easy and rapid for routine use. The actual technique employed, a modification of that originally described by Mirsky & Pollister (1946), was as follows.

The entire procedure was carried out at 0 - 4°C. In order to obtain an adequate number of nuclei for analysis (approximately 400 million) it was generally desirable to start with about 5 g. of tissue (e.g., the liver or intestine of a single rat or the pooled spleens or kidneys of 4 - 8 animals). Where necessary, however, the method could be applied to smaller amounts of material. The tissue was immersed in about 2 volumes of 0.05 M citric acid, and if it had been frozen hard for storage, was allowed to thaw before being minced finely with scissors. The mince and citric acid were then transferred to a Waring, Atomix or Nelco Blendor fitted with an ice-jacket. Further 0.05 M citric acid was added to cover the blades of the Blendor and the tissue was homogenized until the homogenate contained only free nuclei and cytoplasmic granules with no whole cells. In the case of liver this could generally be achieved by a 5-minute run at full speed in the Waring Blendor or 5 minutes at half speed in the Atomix or 3 minutes at full speed in the Nelco. It should be emphasized that these times and speeds

are by no means critical and could probably be exceeded with safety, since, as has already been pointed out (Section 1.4), nuclei isolated in citric acid are remarkably resistant to mechanical damage. The time of homogenization was increased for tissues which are tougher or more fibrous than liver (e.g., kidney and heart muscle). Where it was necessary to isolate nuclei from small amounts of tissue, as for example in the case of thymus and pancreas, a Potter-Elvehjem type homogenizer fitted with a perspex pestle was employed (Potter & Elvehjem, 1936).

After addition of a few drops of capryl alcohol to reduce the foam produced during homogenization the homogenate was filtered through a double layer of gauze to remove any fragments of tissue which might have escaped disintegration in the Blendor and centrifuged at 650 g. for 10 minutes to sediment the nuclei. The supernatant was discarded, the nuclei resuspended in 0.01 M citric acid and the suspension centrifuged at 370 g. for 10 minutes. The supernatant was again discarded. The nuclei were then repeatedly resuspended in 0.01 M citric acid and centrifuged down until on microscopic examination of an unstained wet preparation they appeared to be reasonably free of cytoplasmic contamination. In the case of liver nuclei this was generally achieved after two or three 5-minute centrifugations at 370 g. Finally,

the nuclei were resuspended in about 12 ml. of 0.01 M citric acid and the suspension filtered through a double layer of fine nylon gauze. The yield of nuclei obtained by this procedure was generally between 20% and 50% of the nuclei originally present in the tissue. In dealing with tissues such as lung, kidney and salivary gland it was sometimes found that the homogenate contained fragments of fibrous material, unbroken by prolonged homogenization. This material could, however, generally be removed by filtration through four layers of fine nylon gauze or, where this proved ineffective, by discarding the upper layer of the sediment obtained on centrifugation.

Estimations of numbers of nuclei. The number of nuclei per ml. in each preparation was estimated by counting in a haemocytometer of the Thoma or Neubauer type using 0.01 M citric acid as diluting fluid. At least 1600 nuclei were counted by the author and another observer working independently.

Isolation of leucocytes. Leucocytes were separated from the pooled citrated blood of about 12 rats by centrifuging in constricted centrifuge tubes according to the method described by Butler & Cushman (1940). They were suspended in 0.01 M citric acid and counted in a haemocytometer in the same way as isolated nuclei.

Analysis of isolated nuclei. The isolated nuclei were fractionated by a modification of the method of Schmidt & Thannhauser (1945). A 5 or 10 ml. aliquot of the counted suspension of nuclei was pipetted into a 15 ml. graduated centrifuge tube, 0.5 vol. of 30% ($^{W}/_{V}$) trichloracetic acid was added to precipitate proteins, and the tube stored overnight in a refrigerator at -10 to -15°C. The following morning the contents of the tube were allowed to thaw and the precipitate was centrifuged down and washed three times with 3 ml. portions of 10% ($^{W}/_{V}$) trichloracetic acid. It was then extracted with 5 ml. portions of, successively, acetone, ethanol, ethanol-chloroform mixture (2:3) at 70°C, ethanol-ether mixture (3:1) at 70°C twice, and ether. The dry acid-insoluble, non-lipid residue was incubated for 18 hours at 37°C with 3 ml. N sodium hydroxide in order to hydrolyse the RNA present to acid-soluble nucleotides (Schmidt & Thannhauser, 1945). The alkaline digest was then cooled in an ice-bath and acidified by addition of 1.5 ml. ice-cold 2.5 N hydrochloric acid and 2.5 ml. 30% ($^{W}/_{V}$) trichloracetic acid. This acidification precipitated DNA together with a large amount of protein while leaving the hydrolysis products of RNA in solution (Schmidt & Thannhauser, 1945). After standing for 30 minutes in the ice-bath to ensure complete precipitation, the precipitate was centrifuged down and washed twice with 1 ml.

portions of ice-cold 10% ($^{W}/_{V}$) trichloracetic acid. The supernatant and washings, which contained the hydrolysis products of the RNA, were combined to form the Second Acid-Soluble Fraction. The precipitate was washed once with 0.2 ml. ice-cold distilled water to remove excess trichloracetic acid, redissolved in 2 ml. N sodium hydroxide, and made up with distilled water to a final volume of 10 ml. (i.e., to a final concentration with respect to sodium hydroxide of 0.2 N). This constituted the Residual Fraction.

DNA-phosphorus (DNAP) was estimated by duplicate

(a) phosphorus determinations,

(b) deoxypentose determinations and

(c) ultraviolet absorption measurements

on the Residual Fraction. RNA-phosphorus (RNAP) was estimated by a phosphorus determination on the Second Acid-Soluble Fraction.

Analysis of whole tissue samples. Whole tissue samples were analyzed by the method of Schmidt & Thannhauser (1945) as modified by Leslie & Davidson (1951). The weighed tissue was transferred quantitatively to a conical 15 ml. centrifuge tube containing 5 ml. ice-cold 10% ($^{W}/_{V}$) trichloracetic acid and thoroughly macerated with a glass rod rotated at high speed by a small electric motor. The precipitate of protein, nucleic acid, etc., was centrifuged

down and washed twice with 5 ml. portions of ice-cold 10% ($^{W}/_{V}$) trichloracetic acid to remove acid-soluble material. The residue was extracted with 5 ml. portions of, successively, 80% ethanol, 100% ethanol, ethanol-chloroform mixture (3:1) at 70°C three times, and ether. These extracts were combined to form the Lipid Fraction. As in the case of the nuclear preparations, the acid-insoluble, lipid-free residue was incubated for 18 hours at 37°C with N sodium hydroxide and the alkaline digest fractionated with 2.5 N HCl and 30% ($^{W}/_{V}$) trichloracetic acid into a Second Acid-Soluble Fraction and a Residual Fraction.

Lipid phosphorus (LP) was estimated by a phosphorus determination on the Lipid Fraction. RNAP and DNAP were estimated by phosphorus determinations on the Second Acid-Soluble and Residual Fractions, respectively. Protein nitrogen (PN) was estimated by determining the nitrogen content of the alkaline digest by the micro-Kjeldahl method as modified by Ma & Zuazaga (1942) and subtracting the amount of nitrogen due to nucleic acid. This was calculated by multiplying the total nucleic acid phosphorus (RNAP + DNAP) by 1.7.

Ionophoretic analysis. It has recently been shown that, in addition to the hydrolysis products of RNA, the Second Acid-Soluble Fraction of the Schmidt & Thannhauser (1945) fractionation contains a small amount of inorganic

phosphorus derived from phosphoprotein (Davidson, Frazer & Hutchison, 1951) and small amounts of non-nucleotide organic phosphorus (Smellie & Davidson, 1951; Davidson & Smellie, 1952). Figures obtained for RNAP by the method described above must therefore be regarded as approximate. In order to determine how large and how variable the error from this source might be an experiment was carried out in which the individual nucleotides of the Second Acid-Soluble Fraction were determined by the paper-ionophoresis method of Davidson & Smellie (1952).

Determination of phosphorus. Phosphorus was determined by a modification of the method of Allen (1940). The reagents used were as follows:

A 10 N sulphuric acid.

B 100 vol. hydrogen peroxide (M.A.R.).

C "Amidol reagent". 1 g. amidol (2:4-diaminophenol hydrochloride) dissolved in 100 ml. of a stock 20% ($^{W}/_{V}$) solution of sodium metabisulphite.

D 8.3% ($^{W}/_{V}$) ammonium molybdate solution containing a few drops of ammonium hydroxide.

An aliquot of the fraction to be analysed containing between 20 and 200 µg. of phosphorus was digested with 1.2 ml. 10 N sulphuric acid and a few drops of 100 vol. hydrogen

peroxide in a micro-Kjeldahl flask. When digestion was complete the flask was allowed to cool and 6.35 ml. distilled water were added followed by 2 ml. amidol reagent and 1 ml. 8.3% ($W/_V$) ammonium molybdate and a further 15 ml. distilled water. The intensity of the blue colour which developed was measured between 10 and 30 minutes after the addition of the reagents in a Hilger "Spekker" photoelectric absorptiometer fitted with Ilford red filters (No.608). Where the amount of phosphorus to be determined was less than 20 µg. $\frac{1}{2}$-quantities or $\frac{1}{5}$-quantities of the reagents were used. A calibration curve for the method was prepared using serial dilutions of a standard solution of potassium dihydrogen phosphate.

Estimation of DNA by deoxypentose determination.

The method employed was based on that originally described by Dische (1930) and subsequently modified by Davidson & Waymouth (1944). The following reagents were used:

A 0.4N hydrochloric acid

B "diphenylamine reagent", prepared by dissolving 1 g. diphenylamine (twice recrystallized from ethanol) in 2 ml. concentrated sulphuric acid and diluting with glacial acetic acid to a final volume of 100 ml.

1 ml. of the Residual Fraction, diluted if necessary with 0.2 N sodium hydroxide so that it contained 15 - 100 µg.

DNAP per ml., was mixed in a test-tube with 1 ml. 0.4 N hydrochloric acid and the mixture heated in a boiling water-bath for 15 minutes to hydrolyze the DNA. After cooling, 4 ml. diphenylamine reagent were added and the solutions thoroughly mixed and replaced in the water bath for a further 6 minutes. After cooling, the intensity of the colour developed was measured in a Hilger "Spekker" photo-electric absorptiometer fitted with Ilford yellow filters (No.606). A reagent blank was also prepared, in which the 1 ml. of Residual Fraction was replaced by 1 ml. of 0.2 N sodium hydroxide. A calibration curve for the method was prepared using serial dilutions of a solution of purified calf thymus DNA the concentration of which, in terms of DNAP per ml., had previously been determined.

Estimation of DNA by ultraviolet absorption measurements. The Residual Fraction to be analysed was diluted with distilled water so that the final concentration of DNAP was between 0.4 and 4.0 µg. per ml. The extinction of this diluted solution was measured at 260 and 290 mµ. in a Beckman DU spectrophotometer or a Unicam SP 500 quartz spectrophotometer, using 0.2 N sodium hydroxide similarly diluted with distilled water as a blank. The difference between the two extinctions was assumed to be proportional to the amount of DNA present. A calibration curve was prepared for this method using, as in the previous method,

serial dilutions of a solution of purified calf thymus DNA of known DNAP concentration.

Tests of statistical significance. Statistical analyses of the results obtained in the present series of experiments will be found in Section 2.6 (following p.124). Students "t" test was used to assess the significance of the difference between the mean values of two groups of data. Where more than two groups of data were compared analysis of variance (Snedecor, 1946; Brownlee, 1948) was used. The conventional notation, $P < 0.05$, $P < 0.02$, $P < 0.01$, $P < 0.001$ is used below to indicate significance on the 5%, 2%, 1% and 0.1% levels respectively. In some of the Tables the conventional abbreviations S.D. and S.E. are used to signify standard deviation and standard error of the mean respectively.

2.3 Results.

DNA content per nucleus in different tissues. The mean values for the DNA content of the nuclei of various rat tissues are shown in Table 20 expressed in terms of picograms (pg.) DNAP per nucleus (1 pg. = 10^{-12} g.). The agreement between the three methods of estimation is reasonably good although figures obtained by the deoxypentose method tend to be higher than those found by the other two methods.

Table 20

Mean values for the nucleic acid content, in terms of deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP), of cell nuclei isolated from tissues of male albino rats on stock diet

(Results are given ± S.E. The figures in brackets represent the number of observations. Adult rats weighed 195–250 g.; young rats weighed 35–90 g.)

Tissue	Stage of development of animals	DNAP (pg./nucleus) as determined by: Phosphorus estimation	Deoxypentose estimation	Ultraviolet absorption	Range of RNAP (pg./nucleus)
Kidney	Adult	0·652 ± 0·0202 (8)	0·664 ± 0·0284 (7)	0·663 ± 0·0145 (8)	0·088–0·211
	Young	0·654 ± 0·0167 (3)	0·702 ± 0·0440 (2)	0·636 ± 0·0077 (3)	0·055–0·139
Spleen	Adult	0·633 ± 0·0247 (8)	0·662 ± 0·0454 (8)	0·634 ± 0·0281 (8)	0·057–0·156
	Young	0·685 ± 0·0278 (3)	0·726 ± 0·0040 (2)	0·694 ± 0·0455 (3)	0·055–0·163
Lung	Adult	0·651 ± 0·0311 (6)	0·692 ± 0·0388 (5)	0·647 ± 0·0283 (6)	0·031–0·262
	Young	0·595 (1)	—	0·595 (1)	0·067
Small intestine	Adult	0·738 ± 0·0175 (4)	0·776 ± 0·0157 (4)	0·684 ± 0·0250 (4)	0·136–0·191
	Young	0·728 (1)	—	0·712 (1)	0·244
Salivary gland	Adult	0·733 ± 0·0170 (2)	—	0·663 ± 0·0110 (2)	0·106–0·258
	Young	0·637 (1)	—	0·626 (1)	0·208
Leucocytes	Adult	0·641 ± 0·0508 (4)	—	0·661 (1)	—
Heart	Adult	0·627 (1)	—	0·689 (1)	—
Bone marrow	Adult	0·670 (1)	—	—	—
Pancreas	Adult	0·712 ± 0·0010 (2)	—	0·726 ± 0·0050 (2)	—
Thymus	Young	0·718 ± 0·0215 (4)	0·719 ± 0·0450 (4)	0·660 ± 0·0365 (4)	0·050–0·127
Liver	Adult	0·913 ± 0·0115 (38)	0·928 ± 0·0190 (30)	0·870 ± 0·0157 (38)	0·103–0·232
	Young	0·758 ± 0·0198 (13)	0·788 ± 0·0267 (11)	0·759 ± 0·0152 (13)	0·134–0·251
	Embryo	0·780 ± 0·0717 (4)	0·841 ± 0·0850 (4)	0·721 ± 0·0763 (4)	0·108

The values obtained for the non-hepatic tissues are of the order of 0.65 - 0.70 pg. DNAP per nucleus (equivalent to 6.7 - 7.2 pg. DNA) although small intestine in young animals, and salivary gland, small intestine and pancreas in adults all give slightly higher figures. Analysis of variance, however, shows that no matter which of the three methods of estimating DNA is used there is no significant different between the mean values found for the different non-hepatic tissues of either the young or the adult rat (see Tables 28, 29, 30, 31, 32 and 33).

Liver nuclei from adult rats, on the other hand, give a value of the order of 0.9 pg. DNAP (equivalent to 9.3 pg. DNA), which differs very significantly from the values obtained for the non-hepatic tissues (see "t" tests for each of the three methods of estimation shown in Tables 34, 35 and 36). The corresponding figures for young rats and embryos are, however, much lower and closer to those for the non-hepatic tissues. The difference between the young and adult animals in respect of average DNAP content per nucleus in the liver is highly significant, irrespective of the method used to estimate DNA (see Table 37). The corresponding difference between embryos and adults is significant in the case of the phosphorus and ultraviolet absorption methods of estimating DNA but not in the case of

Table 21

Mean values for the nucleic acid content, in terms of deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP), of rat liver nuclei

(Results are given ± S.E. except where stated. The figures in brackets represent the number of observations.)

Treatment of animal	Strain and sex	Body wt. (g. ± S.D.) Initial	Body wt. (g. ± S.D.) Final	DNAP (pg./nucleus) as determined by: Phosphorus estimation	Deoxypentose estimation	Ultraviolet absorption	RNAP (pg./nucleus)
Section 1:							
All animals on stock diet (*a*)	Albino, M.	—	221 ± 14 (43)	0·913 ± 0·0115 (38)	0·928 ± 0·0189 (30)	0·870 ± 0·0157 (38)	0·180 ± 0·00639 (23)
	Albino, M.	—	271 ± 12 (14)	0·873 ± 0·0287 (14)	0·861 ± 0·0382 (11)	0·844 ± 0·0294 (12)	0·208 ± 0·0343 (5)
	Albino, F.	—	199 ± 11 (14)	0·942 ± 0·0397 (10)	1·004 ± 0·0378 (6)	0·912 ± 0·0557 (9)	0·172 ± 0·0117 (9)
	Albino, F. (pregnant)	—	234 ± 17 (12)	0·893 ± 0·0222 (12)	0·919 ± 0·0396 (10)	0·832 ± 0·0275 (12)	0·181 ± 0·0134 (3)
	Hooded, M.	—	215 ± 12 (12)	0·921 ± 0·0248 (12)	0·965 ± 0·0371 (12)	0·855 ± 0·0274 (12)	0·182 ± 0·0148 (10)
	Hooded, M.	—	291 ± 15 (13)	0·903 ± 0·0316 (13)	0·942 ± 0·0352 (13)	0·851 ± 0·0287 (13)	0·188 ± 0·0881 (12)
	Hooded, F.	—	215 ± 13 (11)	0·931 ± 0·0333 (10)	0·951 ± 0·0606 (10)	0·890 ± 0·0567 (10)	0·169 ± 0·0850 (7)
Section 2. Albino rats of initial wt. 200–250 g. on various diets:							
Stock diet (*a*)	Albino, M.	—	221 ± 14 (43)	0·913 ± 0·0115 (38)	0·928 ± 0·0189 (30)	0·870 ± 0·0157 (38)	0·180 ± 0·00639 (23)
Fast (72 hr.) after diet (*a*)	Albino, M.	224 ± 12 (7)	190 ± 11 (7)	0·885 ± 0·0392 (6)	1·026 ± 0·0210 (3)	0·860 ± 0·0245 (5)	0·144 ± 0·0134 (6)
High-fat diet for 14 days	Albino, M.	223 ± 7 (17)	198 ± 17 (17)	0·889 ± 0·0229 (15)	0·946 ± 0·0372 (10)	0·871 ± 0·0293 (14)	0·169 ± 0·0103 (11)
High-fat diet for 35 days	Albino, M.	217 ± 6 (3)	184 ± 11 (3)	0·902 ± 0·0315 (3)	0·937 ± 0·0547 (3)	0·848 ± 0·0313 (3)	0·160 ± 0·00658 (3)
Semi-synthetic diet (*b*)	Albino, M.	—	223 ± 12 (16)	0·893 ± 0·0294 (12)	0·919 ± 0·0304 (11)	0·852 ± 0·0279 (12)	0·173 ± 0·0200 (6)
Fast (48 hr.) after diet (*b*)	Albino, M.	219 ± 7 (14)	205 ± 11 (14)	0·916 ± 0·0191 (13)	0·943 ± 0·0280 (10)	0·883 ± 0·0244 (13)	0·155 ± 0·0104 (7)
Protein-free diet (*c*) for 7 days	Albino, M.	223 ± 6 (13)	199 ± 10 (13)	0·943 ± 0·0386 (9)	0·990 ± 0·0628 (7)	0·908 ± 0·0424 (9)	0·197 ± 0·0152 (8)
Protein-free diet (*c*) for 15 days	Albino, M.	217 ± 8 (12)	180 ± 15 (12)	0·872 ± 0·0308 (12)	0·897 ± 0·0366 (11)	0·837 ± 0·0320 (12)	0·183 ± 0·0154 (8)
Thiamine-deficient diet (*d*) for 21 days	Albino, M.	228 ± 20 (10)	196 ± 17 (10)	0·912 ± 0·0380 (7)	0·917 ± 0·0594 (6)	0·868 ± 0·0376 (7)	0·141 ± 0·0119 (4)
Thioacetamide-containing diet (*e*) for 7 days	Albino, M.	215 ± 12 (4)	204 ± 18 (4)	0·917 ± 0·0582 (4)	0·934 ± 0·0748 (3)	0·894 ± 0·0683 (4)	0·263 ± 0·0314 (4)
Section 3:							
Alloxan-diabetic rats	Albino, M.	216 ± 8 (5)	187 ± 16 (5)	0·891 ± 0·0125 (5)	0·976 ± 0·0346 (4)	0·888 ± 0·0134 (5)	0·147 ± 0·00734 (5)
Controls for alloxan-diabetic rats	Albino, M.	212 ± 2 (4)	213 ± 6 (4)	0·874 ± 0·0305 (4)	0·886 ± 0·0365 (3)	0·879 ± 0·0332 (4)	0·147 ± 0·00805 (4)

the deoxypentose method (see Table 38). None of the three methods of estimating DNA reveals a significant difference in average DNAP content per nucleus between the livers of the young rats and the embryos (see Table 39).

DNA content per nucleus in liver tissue. Table 21, Section 1 shows the effects of sex, strain, body weight and pregnancy in the DNAP content of the liver nuclei. The mean values for the different groups of animals fall very close together and analysis of variance for each of the three methods of estimation does not indicate that the slight differences between them are significant (see Tables 40, 41 and 42).

The effect of various dietary treatments on the average DNAP content of the liver nuclei is shown in Table 21, Section 2. Once again analysis of variance for each of the three methods of estimation does not indicate that the small differences between the means for the different groups of animals are significant (see Tables 43, 44 and 45). The effect of a diabetogenic dose of alloxan is shown in Table 21, Section 3. The values of "t" found for each of the three methods of estimation (see Table 46) do not indicate a significant change in the average DNAP content of the liver nuclei, although Diermeier et al. (1951) have reported that, in the rat, alloxan caused a 12% increase in the DNA content of round liver nuclei of about 7 μ. diameter.

RNA content of isolated nuclei. Figures for the average RNAP content of the isolated nuclei are also presented in Tables 20 and 21. In Table 20 they are expressed as a range since only a few results were available for each tissue and these showed a wide scatter. The results obtained for liver nuclei were rather more numerous and consistent and are therefore expressed as mean values with standard errors (Table 21). They must, however, be interpreted with considerable caution since although nuclei are known to contain some RNA (see Section 1.7 above) this constitutes only a small proportion of the total RNA of the tissue (see especially Vendrely & Vendrely, 1948; Barnum et al., 1950; Allfrey et al., 1952; Leuchtenberger, Leuchtenberger, Vendrely & Vendrely, 1952; McIndoe & Davidson, 1952; Mauritzen et al., 1952). The apparent RNA content of isolated nuclei will therefore be substantially increased if they are contaminated with cytoplasmic débris. Nevertheless, it is of interest to observe that fasting appears to cause a decrease in the average RNA content of the nuclei and that (in agreement with Laird, 1952) thioacetamide appears to cause an increase.

Composition of whole liver tissue. The results of the analyses of whole liver tissue are shown in Table 22 expressed as

Table 22

Table 22

Mean composition of rat liver tissue in terms of lipid phosphorus (LP), protein nitrogen (PN), ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP)

(Results are given ± S.E. except where stated. The figures in brackets are the number of observations.)

Treatment of animal	Strain and sex	Body wt. (g. ± S.D.) Initial	Body wt. (g. ± S.D.) Final	Liver wt. (g.)	LP (mg./100 g. liver)	PN (mg./100 g. liver)	RNAP (mg./100 g. liver)	DNAP (mg./100 g. liver)	LP (mg./liver)	PN (mg./liver)	RNAP (mg./liver)	DNAP (mg./liver)	LP (pg./pg. DNAP)	PN (pg./pg. DNAP)	RNAP (pg./pg. DNAP)	Tissue mass (pg./pg. DNAP)
Section 1. Stock diet (*a*)	Albino, M.	—	212 ± 8 (24)	7·67 ± 0·190 (23)	132·8 ± 3·31 (23)	2523 ± 64·1 (14)	93·2 ± 1·55 (23)	21·6 ± 0·664 (23)	10·21 ± 0·278 (23)	194 ± 5·21 (14)	7·19 ± 0·197 (23)	1·66 ± 0·0534 (23)	6·28 ± 0·264 (23)	120·3 ± 4·93 (14)	4·38 ± 0·120 (23)	4710 ± 130 (23)
	Hooded, M.	—	215 ± 12 (12)	7·28 ± 0·262 (12)	142·6 ± 4·30 (11)	2618 ± 55·7 (11)	91·1 ± 1·89 (11)	22·9 ± 0·884 (11)	10·29 ± 0·415 (11)	189 ± 3·05 (11)	6·55 ± 0·218 (11)	1·63 ± 0·0645 (11)	6·40 ± 0·368 (11)	116·3 ± 6·25 (11)	4·04 ± 0·148 (11)	4480 ± 216 (11)
	Albino, M.	—	276 ± 10 (10)	8·53 ± 0·212 (10)	127·7 ± 3·56 (10)	2401 ± 53·0 (6)	90·3 ± 3·81 (10)	21·0 ± 1·01 (10)	10·91 ± 0·459 (10)	208 ± 2·24 (6)	7·69 ± 0·338 (10)	1·79 ± 0·0872 (10)	6·22 ± 0·346 (10)	112·5 ± 4·97 (6)	4·32 ± 0·0885 (10)	4880 ± 272 (10)
	Hooded, M.	—	281 ± 9 (8)	8·83 ± 0·401 (8)	141·1 ± 4·70 (8)	2545 ± 95·8 (8)	94·0 ± 3·62 (8)	22·2 ± 0·940 (8)	12·43 ± 0·632 (8)	224 ± 9·86 (8)	8·31 ± 0·546 (8)	1·96 ± 0·120 (8)	6·41 ± 0·249 (8)	115·8 ± 5·79 (8)	4·25 ± 0·112 (8)	4560 ± 186 (8)
	Albino, F.	—	207 ± 8 (8)	6·55 ± 0·348 (8)	123·9 ± 2·89 (8)	2522 ± 81·3 (3)	99·9 ± 2·80 (8)	27·4 ± 0·479 (8)	8·06 ± 0·318 (8)	158 ± 7·39 (3)	6·56 ± 0·439 (8)	1·80 ± 0·0980 (8)	4·52 ± 0·113 (8)	91·7 ± 5·97 (3)	3·64 ± 0·0880 (8)	3650 ± 61·9 (8)
	Hooded, F.	—	215 ± 13 (11)	6·96 ± 0·295 (11)	125·5 ± 2·44 (11)	2598 ± 76·5 (11)	99·7 ± 2·98 (11)	27·7 ± 1·26 (11)	8·68 ± 0·306 (11)	180 ± 9·25 (11)	6·94 ± 0·363 (11)	1·93 ± 0·116 (11)	4·49 ± 0·235 (11)	92·2 ± 4·51 (11)	3·51 ± 0·0805 (11)	3670 ± 145 (11)
Section 2. Stock diet (*a*)	Albino, F.	—	199 ± 11 (14)	6·59 ± 0·266 (14)	127·1 ± 2·14 (14)	2566 ± 55·1 (8)	98·6 ± 1·71 (14)	26·9 ± 0·406 (14)	8·33 ± 0·312 (14)	159 ± 8·26 (8)	6·51 ± 0·332 (14)	1·77 ± 0·0698 (14)	4·73 ± 0·109 (14)	95·4 ± 2·93 (8)	3·67 ± 0·0710 (14)	3720 ± 58·0 (14)
	Albino, F. (pregnant)	—	234 ± 17 (11)	7·65 ± 0·258 (11)	135·0 ± 3·80 (10)	2531 ± 61·5 (8)	114·9 ± 4·22 (10)	24·4 ± 1·17 (10)	10·24 ± 0·372 (10)	192 ± 9·81 (8)	8·69 ± 0·276 (10)	1·84 ± 0·0708 (10)	5·61 ± 0·212 (10)	106·8 ± 4·90 (8)	4·79 ± 0·244 (10)	4180 ± 182 (10)
	Albino (embryo)	—	—	—	60·48 ± 3·52 (4)	—	89·9 ± 3·46 (4)	45·9 ± 3·46 (4)	—	—	—	—	1·33 ± 0·083 (4)	—	1·98 ± 0·140 (4)	2220 ± 161 (4)
	Albino, M.	—	35–90	—	121·5 ± 3·09 (13)	2358 ± 39·2 (6)	100·0 ± 2·48 (13)	25·6 ± 0·799 (13)	—	—	—	—	4·79 ± 0·146 (13)	89·9 ± 2·32 (6)	3·93 ± 0·0805 (13)	3960 ± 137 (13)
Section 3.																
Stock diet (*a*)	Albino, M.	—	221 ± 14 (43)	7·94 ± 0·149 (43)	131·4 ± 2·24 (41)	2469 ± 47·7 (27)	91·8 ± 1·32 (41)	21·9 ± 0·479 (41)	10·51 ± 0·213 (41)	198 ± 4·78 (27)	7·35 ± 0·160 (41)	1·75 ± 0·0458 (41)	6·14 ± 0·182 (41)	116·0 ± 3·57 (27)	4·27 ± 0·0925 (41)	4660 ± 93·0 (41)
Fast (72 hr.) after stock diet	Albino, M.	224 ± 12 (7)	190 ± 11 (7)	4·89 ± 0·158 (7)	141·4 ± 2·98 (7)	2955 ± 4·95 (2)	109·1 ± 4·86 (7)	33·4 ± 1·64 (7)	6·91 ± 0·266 (7)	150 ± 8·49 (2)	5·33 ± 0·292 (7)	1·63 ± 0·0914 (7)	4·30 ± 0·246 (7)	87·1 ± 2·30 (2)	3·28 ± 0·0987 (7)	3030 ± 148 (7)
High-fat diet for 14 days	Albino, M.	223 ± 7 (17)	198 ± 17 (17)	9·01 ± 0·455 (17)	89·0 ± 3·14 (17)	1922 ± 74·5 (7)	68·1 ± 2·18 (17)	20·6 ± 0·731 (17)	7·94 ± 0·404 (17)	182 ± 9·22 (7)	6·01 ± 0·206 (17)	1·82 ± 0·0816 (17)	4·27 ± 0·179 (17)	95·4 ± 4·86 (7)	3·33 ± 0·0839 (17)	4960 ± 176 (17)
High-fat diet for 35 days	Albino, M.	217 ± 6 (3)	184 ± 11 (3)	8·40 ± 0·378 (3)	92·1 ± 1·97 (3)	1891 ± 94·0 (3)	67·1 ± 2·38 (3)	20·0 ± 1·43 (3)	7·73 ± 0·256 (3)	158 ± 9·24 (3)	5·63 ± 0·277 (3)	1·67 ± 0·109 (3)	4·64 ± 0·226 (3)	95·0 ± 2·38 (3)	3·37 ± 0·124 (3)	5060 ± 358 (3)
Section 4.																
Semi-synthetic diet (*b*)	Albino, M.	—	223 ± 12 (16)	8·22 ± 0·178 (16)	110·5 ± 2·62 (15)	2380 ± 116·0 (9)	91·9 ± 2·44 (15)	21·1 ± 0·702 (15)	9·00 ± 0·229 (15)	193 ± 8·70 (9)	7·53 ± 0·297 (15)	1·73 ± 0·0762 (15)	5·26 ± 0·157 (15)	116·4 ± 7·80 (9)	4·38 ± 0·0890 (15)	4810 ± 160 (15)
Fast (48 hr.) after diet (*b*)	Albino, M.	219 ± 7 (14)	205 ± 11 (14)	5·53 ± 0·179 (14)	139·8 ± 2·30 (13)	2603 ± 82·6 (8)	107·7 ± 2·33 (13)	33·3 ± 0·899 (13)	7·68 ± 0·200 (13)	146 ± 6·30 (8)	5·93 ± 0·207 (13)	1·83 ± 0·0550 (13)	4·22 ± 0·100 (13)	79·2 ± 3·02 (8)	3·25 ± 0·0631 (13)	3030 ± 84·4 (13)
Protein-free diet (*c*) for 7 days	Albino, M.	223 ± 6 (13)	199 ± 10 (13)	6·30 ± 0·209 (13)	96·8 ± 3·13 (12)	2080 ± 104·1 (8)	81·6 ± 2·37 (12)	25·5 ± 0·650 (12)	6·07 ± 0·228 (12)	133 ± 7·89 (8)	5·11 ± 0·143 (12)	1·60 ± 0·0600 (12)	3·79 ± 0·0545 (12)	81·9 ± 6·40 (8)	3·20 ± 0·0488 (12)	3950 ± 102 (12)
Protein-free diet (*c*) for 15 days	Albino, M.	217 ± 8 (12)	180 ± 15 (12)	5·69 ± 0·302 (12)	101·5 ± 1·99 (12)	1845 ± 82·7 (8)	92·2 ± 1·46 (12)	29·4 ± 0·745 (12)	5·77 ± 0·323 (12)	108 ± 7·21 (8)	5·22 ± 0·247 (12)	1·67 ± 0·0909 (12)	3·46 ± 0·0845 (12)	62·3 ± 1·80 (8)	3·16 ± 0·0894 (12)	3430 ± 90·2 (8)
Thiamine-deficient diet for 21 days	Albino, M.	228 ± 20 (10)	196 ± 17 (10)	5·43 ± 0·207 (10)	130·1 ± 6·33 (10)	2945 ± 135·0 (5)	98·0 ± 2·91 (10)	33·9 ± 1·38 (10)	7·02 ± 0·337 (10)	153 ± 10·80 (5)	5·31 ± 0·212 (10)	1·83 ± 0·0616 (10)	3·86 ± 0·154 (10)	83·4 ± 8·07 (5)	2·93 ± 0·124 (10)	2990 ± 118 (10)
Thioacetamide-containing diet for 7 days	Albino, M.	215 ± 12 (4)	204 ± 18 (4)	7·93 ± 0·688 (4)	111·7 ± 1·68 (3)	2048 ± 19·2 (2)	89·3 ± 2·08 (3)	22·5 ± 0·618 (3)	9·33 ± 0·735 (3)	183 ± 20·5 (2)	7·44 ± 0·480 (3)	1·88 ± 0·1560 (3)	4·98 ± 0·179 (3)	90·3 ± 4·65 (2)	3·98 ± 0·107 (3)	4460 ± 120 (3)
Section 5.																
Alloxan-diabetic rats	Albino, M.	216 ± 8 (5)	187 ± 16 (5)	6·24 ± 0·300 (5)	128·6 ± 4·38 (5)	—	93·5 ± 4·91 (5)	25·7 ± 0·975 (5)	7·98 ± 0·238 (5)	—	5·79 ± 0·206 (5)	1·60 ± 0·0559 (5)	5·01 ± 0·166 (5)	—	3·62 ± 0·0666 (5)	3910 ± 146 (5)
Controls for alloxan-diabetic rats	Albino, M.	212 ± 2 (4)	213 ± 6 (4)	7·60 ± 0·158 (4)	119·3 ± 3·58 (4)	—	86·2 ± 2·75 (4)	23·2 ± 0·975 (4)	9·05 ± 0·161 (4)	—	6·54 ± 0·176 (4)	1·76 ± 0·0690 (4)	5·16 ± 0·170 (4)	—	3·73 ± 0·0610 (4)	4340 ± 182 (4)

(a) concentrations per 100 g. fresh liver,

(b) total amounts in mg. per liver, and

(c) pg. per pg. DNAP.

Since the average DNAP content per nucleus in the liver of the adult rat is about 0.9 pg. irrespective of its sex, strain and body weight, and of the diet on which it has been maintained, the third method of expression gives an approximate estimate of the average cell composition. Similarly, the total DNAP content of the liver in pg. (1 mg. = 10^9 pg.), gives an approximate estimate of the number of cells which it contains. Both these estimates are approximate unless corrections are made (a) for the occurrence of binucleate cells in the liver, and (b) for the fact that a proportion of the liver substance is extracellular. Methods are available by which the magnitude of both these corrections may be determined but unfortunately they are laborious and of rather doubtful accuracy (Harrison, 1953a, b).

The usefulness of this method of expression may be appreciated from a consideration of the differences in liver composition between male and female rats of the same strain and of comparable body weight (Table 22, Section 1). It will be seen that in both the albino and hooded strains there is little difference between the sexes in the concentration of LP, PN, or RNAP per 100 g. liver. The concen-

tration of DNAP, on the other hand, is about 20% higher in females than in males and this difference is significant ($P < 0.01$ in both strains, see Tables 47 and 48). A similar difference has been observed by Lowe & Salmon (1951). The total amount of LP in the liver is significantly lower in females than in males ($P < 0.001$ in both strains, see Tables 47 and 48) but the differences in total amounts per liver of PN, RNAP and DNAP are of dubious significance (see Tables 47 and 48) and are not consistent between the two strains. When, however, the results are referred to DNAP it becomes clear that, in the female, the average mass of the cell, and its average content of LP, PN and RNAP are 15 - 30% lower than in the male and that these differences between the sexes are significant (see Tables 49 and 50). Using a similar method Harrison (1953b) has also found that the liver cells of female rats have a lower average mass than those of males. These results are in agreement with Korenchevsky's histological observation that the mean cell volume in rat liver is greater in the male than in the female (Korenchevsky, Hall, Burbank & Cohen, 1941).

Similar calculations show that in pregnancy (Table 22, Section 2; see Table 51 for statistical analysis) the total amount of DNAP (i.e., number of cells) in the liver

is not significantly altered but the average cell mass increases by 12% ($P < 0.02$), the average cell content of LP by 20% ($P < 0.001$), and of RNAP by 30% ($P < 0.001$). No significance can be attached to the apparent increase in average cell content of PN. These results are in agreement with the finding of Campbell & Kosterlitz (1949) that in the rat pregnancy causes a marked increase in the total RNA of the liver.

Some figures for the composition of liver tissue from young rats and embryos are also included in Table 22, Section 2. The liver of the young animal has a composition not unlike that of the adult, but the embryo liver contains about twice as much DNAP and only about half as much LP per 100 g. fresh weight. Making allowance for the fact that the mean DNAP content per nucleus is lower in this tissue than in adult liver (0.75 pg. as compared with 0.90 pg.) it would appear that the cells in the embryo liver are, on the average, only half as large as those in the adult organ and that they contain only half as much RNAP and a quarter as much LP. In view of the well-known differences in function between the embryo and adult organs (e.g., as McKellar, 1949, has shown, haemopoiesis occurs in the former but not in the latter) these differences in average cell composition are hardly surprising.

Effects of various diets. In considering the effects of the various dietary treatments shown in Sections 3 and 4 of Table 22 it is convenient to consider separately their effect on the total number of cells in the liver and on the average cell composition. It is obvious that none of the treatments has had any marked effect on the total amount of DNAP per liver. The small differences between the means for the different groups have been shown by analysis of variance (Table 52) to be non-significant. It appears, therefore, that none of the treatments has significantly affected the number of cells in the liver. The average cell composition, on the other hand, is drastically changed. On fasting after either the semi-synthetic or the stock diet, the average cell mass falls by about 35% and the average cell content of LP, PN and RNAP by 20 - 30%. These changes appear to be highly significant (see Tables 53 and 54). Since, as has already been pointed out above (Section 2.2), the "RNAP" as estimated by the method used in the present series of experiments includes a certain amount of phosphorus derived from compounds other than RNA it seemed of some importance to determine whether the apparent fall in RNAP produced by fasting was, in fact, due to a real fall in RNA or merely to a fall in the "concomitant phosphorus compounds. Accordingly, the individual nucleo-

Table 23.

Effect of asting on the proportion of the "ribonucleic acid phosphorus" ("RNAP") of rat liver (as determined by the method of Schmidt & Thannhauser, 1945) due to the presence of phosphorus compounds other than ribonucleic acid (RNA).

Condition of animals	Total "RNAP"* per liver mg.	"RNAP" due+ to RNA per liver mg.	"RNAP" due to compounds other than RNA per liver** mg.	"RNAP" due to compounds other than RNA as % of total "RNAP"
Normal controls	7.04	5.09	1.95	27.7%
	7.82	5.35	2.47	36.6%
Fasted 72 hours	5.92	4.31	1.61	27.2%
	4.60	3.22	1.38	30.0%

* As determined by the method of Schmidt & Thannhauser (1945).

\+ As determined by the method of Davidson & Smellie (1952)

** By difference.

tides in the Second Acid-Soluble fractions derived from the livers of two fed and two fasted rats were determined by paper ionophoresis. The results obtained are shown in Table 23. They indicate quite clearly that the RNA and the concomitant phosphorus compounds in this fraction are about equally affected by fasting.

The thiamine-deficient diet produced significant changes in the average composition per cell in the liver similar to those caused by fasting (see Table 55 for statistical analysis). These may, however, be due not so much to the vitamin deficiency itself as to the consequent loss of appetite. The protein-free diet caused a 20% fall in the average cell mass and a 30% fall in the average cell content of LP, PN and RNAP during the first 7 days. These changes were all highly significant (see Table 56 for statistical analysis). During the second 7 days there was a further significant fall in LP, PN and cell mass ($P < 0.01$, $P < 0.02$ and $P < 0.01$, respectively; see Table 57 for statistical analysis) but the RNAP content did not decline further. The high-fat diet caused significant reductions in the LP, PN and RNAP content of the cell similar to those observed in protein deficiency, but less severe (see Table 58 for statistical analysis). This might be expected since the high fat diet contains only 8% protein. The

average cell mass did not fall, and it is presumed that increased deposition of neutral fat balanced the loss of other cytoplasmic constituents.

Although histological examination of liver sections from rats which had received the thioacetamide-containing diet revealed morphological changes similar to those described by Rather (1951) and, in particular, a marked increase in the volumes of the nuclei and nucleoli, this substance does not appear to have had any significant effect on the average composition of the cells of the liver (see statistical analysis in Table 59). Similar findings have been recorded by Laird (1952).

Finally, the animals on the stock diet differed from those on the semi-synthetic diet in having a significantly higher content of LP per cell ($P < 0.01$; see Table 60 for "t" test). The difference disappeared on fasting.

Effect of diabetes. Administration of a diabetogenic dose of alloxan (Table 22, Section 5; see Table 61 for statistical analysis) caused a significant fall in the total mass of the liver ($P < 0.01$) and in its total content of LP and RNAP ($P < 0.01$ and $P < 0.02$, respectively). Since, however, the falls in total content of RNAP (i.e., in number of cells per liver) and in average cell mass and content of LP and RNAP which accompanied these changes are not statistically significant, it is not possible to say

whether the changes in the composition of the whole organ were due to a reduction of cell number or to a change in average cell mass and composition, or to both. The effect of alloxan on the composition of the liver of the rat has also been investigated by Rerábek (1947), who finds that it increases the concentration (in mg. per 100 g. fresh liver) of RNA but not of DNA. These effects were, however, produced by doses of alloxan three or six times as great as those used in the present series of experiments.

Effects of the carcinogenic diet. The effects of the carcinogenic diet on the average DNAP content of the liver nuclei and the composition of whole liver tissue are shown in Table 24. Compared with the controls the animals which received the carcinogen had a lower average content of DNAP per nucleus and a higher concentration of DNAP per 100 g. fresh liver. In those cases in which the total DNA content of the liver was determined, it was generally found to be considerably increased. The total amounts and concentrations per 100 g. of LP, PN and RNAP in the liver were not greatly affected but the ratios of these components to DNAP were markedly lower. Some animals developed tumours sufficiently large and well-defined to be dissected out from the rest of the liver and analyzed separately. Where this was done (rats no.H5, 107-108 and 109-110) it was found

Table 24

Table 24

Mean composition of whole tissue and nuclei, in terms of lipid phosphorus (LP), protein nitrogen (PN), ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP), in the livers of rats fed a diet containing p-*dimethylaminoazobenzene*

(Control animals were kept for the same time on the same diet without the dimethylaminoazobenzene. Except where otherwise indicated analyses and isolation of nuclei were carried out on portions of the whole liver.)

				Composition of whole liver tissue				Composition of whole liver tissue				Composition of isolated nuclei				
												DNAP (pg./nucleus) as determined by				
Treatment and description of animals	Rat number	Body wt. at death (g.)	Liver wt. (g.)	LP (mg./100 g. liver)	PN (mg./100 g. liver)	RNAP (mg./100 g. liver)	DNAP (mg./100 g. liver)	LP (mg./liver)	PN (mg./liver)	RNAP (mg./liver)	DNAP (mg./liver)	LP (pg./pg. DNAP)	PN (pg./pg. DNAP)	RNAP (pg./pg. DNAP)	Tissue mass (pg./pg. DNAP)	Phosphorus estimation	Deoxypentose estimation	Ultraviolet absorption	RNAP (pg./nucleus)	Remarks
Carcinogenic diet for 5 months. Initial body wt. 150–170 g.*	H1	132	7·2	- -	1950	70·6	32·9	-	140	5·08	2·37	-	59·3	2·15	3040	0·802	0·916	0·774	0·138	Diffuse tumour
	H2	136	7·8	-	2200 L	75·0 L	34·8 L	-	- -	- -	—	-	63·2 L	2·16 L	2870 L	0·722	0·832	0·692	0·143	
	H3	105	6·2	—	2000 T	79·4 T	36·0 T	-	- -	- -	—	-	55·5 T	2·21 T	2780 T	0·792	0·859	0·750	0·133	
	H5	147	13·6	-	-	78·8 L	29·2 L	- -	—	—	—	- -	—	2·70 L	3420 L	0·792	—	0·744	0·192	
				-	-	53·8 T	39·2 T	-	—	—	—	-	—	1·37 T	2550 T					
	H6	97	3·3	- -	2340	79·0	37·5	-	77	2·60	1·24	—	62·4	2·11	2670	0·823	1·165	0·824	0·121	No obvious tumour
	H7	183	8·4	-	1980	63·1	28·0	-	166	5·30	2·35	—	70·7	2·25	3570	0·725	0·842	0·669	0·109	
	H8	139	6·8	-	1990	65·0	32·0	-	135	4·42	2·18	—	62·2	2·03	3130	0·721	0·777	0·668	0·105	
	H9	172	7·5	-	1970	68·7	38·5	- -	148	5·15	2·88	—	51·2	1·78	2600	0·700	0·791	0·664	0·108	Diffuse tumours
	H10	170	9·0	-	2110	75·8	31·4	—	190	6·82	2·83	—	67·2	2·41	3180	0·715	0·847	0·671	0·119	
	H11	132	6·9	-	2170	75·0	34·0	-	150	5·18	2·35	—	63·8	2·21	2940	0·731	0·840	0·679	0·096	
Controls for above: four animals of initial body wt. 163–195 g.*	—	159–199	6·80 ± 0·791	-	2380 ± 91	83·3 ± 2·25	25·5 ± 0·851	—	161 ± 16·4	5·64 ± 0·592	1·73 ± 0·203	—	93·8 ± 3·85	3·27 ± 0·082	3940 ± 126	0·951 ± 0·0122	0·974 ± 0·0435	0·907 ± 0·0499	0·190 ± 0·0151	Results expressed as means ± S.E.
Carcinogenic diet for 6 months. Initial body wt. 190–210 g.	107	180	13·6	77·2 T	1950 T	72·7 T	39·9 T	—	—	—	—	1·93 T	48·9 T	1·82 T	2510 T	0·648 T	0·643 T	0·620 T	0·302 T	
	108	200	11·2	105·3 L	2260 L	76·0 L	28·6 L	—	—	—	—	3·67 L	78·8 L	2·66 L	3500 L	0·664 L	0·735 L	0·713 L	0·294 L	
	109	195	7·81	63·1 T	1570 T	68·8 T	44·0 T	—	—	—	—	1·43 T	35·6 T	1·56 T	2270 T	0·651 T	—	0·574 T	0·428 T	
	110	155	20·3	96·4 L	2160 L	64·8 L	24·8 L	—	—	—	—	3·89 L	87·1 L	2·61 L	4030 L	0·687 L	0·710 L	0·677 L	0·303 L	
	111	210	8·4	104·0	2350	78·6	27·3	8·74	197	6·60	2·29	3·81	94·8	2·88	3660	0·729	0·823	0·718	0·255	Liver almost normal in appearance
Controls for above: four animals of initial body wt. 190–210 g.	—	200–243	6·53 ± 0·441	94·7 ± 3·83	2440 ± 38	78·6 ± 3·36	25·8 ± 0·477	6·15 ± 0·291	158 ± 8·1	5·10 ± 0·213	1·68 ± 0·114	3·68 ± 0·143	94·8 ± 5·21	3·05 ± 0·130	3880 ± 73	0·956 ± 0·0462	0·947 ± 0·0339	0·913 ± 0·0471	0·364 ± 0·0198	Results expressed as means ± S.E.
Carcinogenic diet for 2 months. Initial body wt. 190–210 g.	465	149	9·2	97·0	-	81·7	37·0	8·92	—	7·52	3·40	2·62	–	2·21	2700	0·735	—	0·738	0·136	Diffuse tumours
	466	141	7·0	98·4	-	85·3	46·7	7·87	—	6·82	3·74	2·11	–	1·83	2140	—	—	—		
	467	139	6·2	109·5	-	83·3	37·3	6·82	—	5·16	2·31	2·95	—	2·23	2680	0·732	—	—	0·136	

T, tumour tissue only. L, residual liver tissue after removal of tumour nodules.

* Whole tissue analyses carried out by E. B. Smith and G. T. Mills.

Table 25

Mean deoxyribonucleic acid phosphorus (DNAP) content of the nuclei of rat liver regenerating after partial hepatectomy and the composition of whole liver tissue in terms of lipid phosphorus (LP), ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP)

(Results given ± S.E. except where stated. Six animals for each time after operation.)

Time after operation (days)	Body wt. (g. ± S.D.)		Composition of whole liver tissue				DNAP (pg./nucleus) as determined by		
	At operation	At death	Liver wt. (g.)	Total DNAP content liver (mg.)	LP (pg./pg. DNAP)	RNAP (pg. pg. DNAP)	Phosphorus estimation	Deoxypentose estimation	Ultraviolet absorption
1	225 ± 27	220 ± 31	3·02 ± 0·164	0·673 ± 0·0362	5·55 ± 0·204	4·48 ± 0·128	1·018*	1·031*	0·911*
2	232 ± 12	218 ± 8	3·58 ± 0·114	0·769 ± 0·0310	5·91 ± 0·201	4·83 ± 0·208	1·258*	1·355*	1·217*
3	227 ± 22	206 ± 24	3·92 ± 0·282	0·974 ± 0·0463	5·01 ± 0·224	4·98 ± 0·0783	1·022† 1·099†	0·985† 1·019†	1·060† 1·112†
4	222 ± 35	202 ± 24	5·18 ± 0·364	1·224 ± 0·101	5·29 ± 0·110	4·72 ± 0·154	1·385† 1·151†	1·375† 1·243†	1·368† 1·138†
6	231 ± 28	216 ± 17	5·42 ± 0·401	1·403 ± 0·0792	4·80 ± 0·184	4·46 ± 0·149	0·903† 0·975†	0·955† 1·015†	0·905† 0·968†
8	260 ± 33	231 ± 23	5·12 ± 0·273	1·392 ± 0·0952	4·92 ± 0·326	4·61 ± 0·264	1·013† 0·949†	1·000† 0·983†	1·061† 1·016†
10	228 ± 24	227 ± 12	6·15 ± 0·289	1·445 ± 0·0989	4·61 ± 0·250	4·25 ± 0·271	0·906‡ 1·014‡ 1·056‡	0·907‡ 1·059‡ 1·200‡	0·901‡ 1·064‡ 1·047‡

* Pooled tissue from six rats. † Pooled tissue from three rats. ‡ Pooled tissue from two rats.

that the tumour exhibited to an exaggerated degree the changes described above. The average DNAP content per nucleus was of the same order as the value found for the non-hepatic tissues of the normal animal. The ratios of LP, PN, RNAP and tissue mass to DNAP were only half as great as in the controls. In the portion of the liver which remained after removal of the tumour the average DNAP content per nucleus was also very low but the ratios of LP, PN, RNAP and tissue mass to DNAP were closer to the normal values. Making allowance for the fact that the average DNAP content per nucleus was lower than in normal liver, these observations might be interpreted as follows:-

(i) The carcinogen caused an increase in the total number of cells in the liver of the order of 50 - 150%.

(ii) The new tumour cells were less than half the average size of the cells of the normal liver. They contained, on the average, only 35% as much LP, 35% as much PN and 40% as much RNAP as normal liver cells.

Effect of hepatectomy. The results of the hepatectomy experiment are shown in Table 25. Throughout the 10 days following the operation the average DNAP content of the nuclei in the remaining lobes of the liver was consistently above the average value for unoperated animals

of the same sex, strain, and age (Table 21). The total amount of DNAP in the liver rose steadily up to the 6th day after the operation. During this phase of rapid growth the average DNAP content per nucleus was particularly high and there were variations in the ratios of LP and RNAP to DNAP. From the 6th to the 10th day therewas no further increase in the total DNAP content of the liver, the values found for the average DNAP content per nucleus were only slightly above the normal figure, and the ratios of LP and RNAP to DNAP showed little change. These results are in agreement with the observations of Price & Laird (1950) and Ultman, Hirschberg & Gellhorn (1953) that the average DNA content per nucleus in the liver of the rat shows a marked increase immediately after hepatectomy and then returns to a value slightly above that found in normal unoperated animals.

2.4 Discussion

The values found for the average DNAP content per nucleus in the various rat tissues examined (Table 20) are in fairly good agreement with those obtained by other workers using the same method, viz., chemical analysis of suspensions of known numbers of isolated nuclei (see Table 26). Cunningham, Griffin & Luck (1950) have devised a modification of this method in which the tissue is homogenized in 2% citric acid, the homogenate is filtered through cheese-

Table 26.

Average deoxyribonucleic acid (DNA) content of nuclei isolated from rat tissues, as reported by various authors. See also Tables 18 and 27.

Tissue	pg. DNA per nucleus	Reference
Liver	10.2 - 11.1	Dounce et al. (1950)
	8	Mirsky & Kurnick (unpublished results cited by Mirsky & Ris, 1951)
	9.8 - 10.2	Ely & Ross (1951a)
	9.6 - 10.2	Harrison (1951)
	8.2	Leuchtenberger et al. (1951)
	8.6	Leuchtenberger et al. (1952)
	9.6	Villela (1952)
Intestine	6.27	Ely & Ross (1951b)
Kidney	5.7	Harrison (1951)
	5.5	Leuchtenberger et al. (1951)
	8	Mirsky & Kurnick (unpublished results cited by Mirsky & Ris, 1951)
	9.3*	Kurnick (1951).

*Recalculated from figures given for content of DNAP per nucleus.

cloth, and the number of nuclei present is estimated by counting. About 70% of the nuclei are sedimented by centrifuging at low speed and washed once with citric acid. The total number of nuclei in the pooled supernatants is estimated by counting. Subtraction gives the number of nuclei in the sediment, which is then analysed for DNA. It is claimed that in this way errors due to the presence of chromatin threads and to clumping of the nuclei may be avoided. On the other hand, the necessity for estimating the number of nuclei in both the original homogenate and the combined supernatants must considerably increase the errors due to the inaccuracy of the counting technique. The results obtained by Cunningham et al. (1950) using this technique (see Table 18) are nevertheless in good agreement with those obtained in the present investigation. Recently the same method has been used by Sibatani and his colleagues in a series of experiments on rat liver nuclei (Sibatani, Matsuda, Fukuda & Naora, 1952; Fukuda & Sibatani, 1953). These workers find the average DNA content per nucleus in the liver to be about 9 pg. in young animals (body weight, 50 g.) and about 10 - 11 pg. in adults (body weight, 200 - 300 g.). These results also are in moderately good agreement with those obtained in the present investigation.

Price and his associates (Price & Laird, 1950; Price, Miller, Miller & Weber, 1950) have employed a quite different method for estimating the average DNA content per nucleus in rat liver. This consists essentially of estimating (a) the concentration of DNA per 100 g. by chemical analysis of a weighed portion of tissue and (b) the number of nuclei per 100 g. by counting the nuclei in a citric acid homogenate of a second weighed portion of the same tissue. Simple division gives the average DNA content per nucleus. The results obtained by this method (see Table 27) are about 10 - 40% greater than those usually found by direct analysis of isolated nuclei. The reason for the discrepancy between the results of the two methods is not known but it is possible that the estimates of the number of nuclei per 100 g. liver used in the method of Price and his associates may be erroneously low due to destruction of nuclei in preparation of the homogenates used for counting. If this is, in fact, the case the values calculated for the DNA content per nucleus will be erroneously high.

Alternatively, the discrepancy might be explained by assuming that the values obtained for DNA content per nucleus by direct analysis of isolated nuclei are erroneously low due to loss of DNA from the nuclei during isolation. On theoretical grounds this seems highly improbable. Since

Table 27.

Deoxyribonucleic acid (DNA) content per nucleus in rat liver as determined by dividing the concentration of DNA per 100 g. by the number of nuclei per 100 g.

Reference	pg. DNA per nucleus
Price & Laird (1950)	10.0
Price et al. (1950)	10.1 - 14.0
Rose & Schweigert (1952)	14.9 - 15.4
Ultman, Hirschberg & Gellhorn (1953)	10.4.

DNA and nucleohistone are both insoluble in dilute citric acid (Dounce, 1943b) DNA could only escape from the nucleus as a result of hydrolysis. It seems inconceivable, however, that DNA, which is quite resistant to extraction for 18 hours with cold N perchloric acid (Ogur & Rosen, 1949, 1950; Koenig & Stahlecker, 1952), should be hydrolysed to any appreciable extent by dilute citric acid. Vendrely (1952) has, in fact, found that calf thymus nucleohistone prepared by the method of Mirsky & Pollister (1943) may be treated with M/3 citric acid for a period of several days without showing any loss of dry weight. Nor is it likely that DNA is hydrolysed by the action of deoxyribonuclease since this enzyme, as McCarty (1946) has shown, requires the presence of calcium ions for activity and these form inactive complexes with citric acid. Direct evidence that nuclei isolated in dilute citric acid do not suffer any loss of DNA in the process has come from the work of Ris & Mirsky (1949a), who have found, using a cytophotometric method, that calf liver nuclei have the same average DNA content after isolation as they do in situ in histological sections, and of Stedman & Stedman (1951) and Vendrely (1952) who have demonstrated by chemical analysis of counted suspensions of nuclei that the average DNA content of avian erythrocyte nuclei is not affected by exposure to dilute

citric acid for a period of several hours.

A more serious criticism which may be levelled at the practice of analysing isolated nuclei is that such nuclei may not constitute a representative sample of the whole population of the nuclei in the tissue from which they were isolated. In rat liver, for example, it might be anticipated that the large hepatocyte nuclei would be more likely to be damaged during homogenization than the small bile duct nuclei. On the other hand, during the differential centrifugation used to separate the nuclei from the cytoplasmic debris the larger nuclei, having a smaller surface area in relation to their mass, may be expected to sediment more rapidly than the smaller nuclei and a disproportionate number of the latter may therefore be discarded with the cytoplasmic debris in the supernatants. This would seem to be a particularly serious source of error in the method of Cunningham et al. (1950) in which only the more rapidly sedimenting fraction of the nuclei is taken for analysis. Until a method is devised whereby nuclei can be isolated from a tissue in 100% yield there does not seem to be any simple means by which sampling errors of this kind may be avoided or their magnitude accurately assessed. Evidence will, however, be presented below which suggests that in the present series of experiments they are probably not very great. In any case, they are

unlikely to be so large as to obscure the answer to the question under investigation, namely, whether all somatic nuclei in the rat contain the same amount of DNA.

The results shown in Table 20 do, in fact, indicate that there is very little variation in average DNA content between nuclei isolated from different non-hepatic tissues, suggesting that all the nuclei in these tissues may contain the same amount of DNA. Isolated liver nuclei, on the other hand, have an average DNA content 30% greater than that found for the nuclei of other tissues. The reason for this anomalously high figure has been revealed by cytophotometric measurements on individual nuclei. These have shown that whereas all the nuclei in rat kidney contain about the same amount of DNA the nuclei of rat liver fall into three classes with respect to their DNA content. "Class I" nuclei contain approximately the same amount of DNA as kidney nuclei. Classes II and III contain twice and four times this amount and it is suggested that they represent tetraploid and octoploid nuclei respectively (Ris & Mirsky, 1949a; Leuchtenberger, Vendrely & Vendrely, 1951; Leuchtenberger, Leuchtenberger, Vendrely & Vendrely, 1952; Frazer & Davidson, 1953). Similar results have been obtained for mouse kidney and liver by Swift (1950a).

It is, of course, well known that polyploid nuclei

are not uncommon in certain mammalian tissues (Teir, 1944). In particular, the relative proportions of diploid, tetraploid and octoploid nuclei in rat liver have been studied by purely histological methods (Beams & King, 1942; Sulkin, 1943; Biesele, 1944; Wilson & Leduc, 1948; McKellar, 1949). The results obtained by these workers are of some interest since they can be used to calculate the theoretical average DNA content of the nuclei in the liver, given the DNA content of the diploid nucleus. For example, Biesele (1944) reported that of 70 mitotic figures which he observed in normal rat liver 54.5% were diploid, 40.5% tetraploid and 5% octoploid. Since all the nuclei of rat kidney contain the same amount of DNA (vide supra) it may be assumed that the average DNAP content per nucleus of about 0.65 pg. found for this tissue in the present investigation is a fairly reliable estimate of the DNAP content of the diploid nucleus. The theoretical average DNAP content per nucleus in the liver should therefore be

$$\frac{(0.65 \times 54.5) + (2 \times 0.65 \times 40.5) + (4 \times 0.65 \times 5)}{100}$$

$$= 1.006 \text{ pg.},$$

which is in reasonably good agreement with the figure of 0.913 pg. found experimentally for the average DNAP content of isolated liver nuclei (Table 20). This calculation is open to the objection that the proportion of polyploid

mitotic figures in a tissue is not necessarily a reliable guide to the proportion of polyploid nuclei since different classes of nuclei may have different mitotic frequencies. However, Sulkin (1943) has estimated on the basis of nuclear volume measurements on histological tissue sections (Jacobj, 1925) that in the rat 12.28% of the hepatocyte nuclei are diploid, 79.18% are tetraploid, 8.14% are octoploid and 0.4% are 16-ploid. Assuming again that the diploid nucleus contains 0.65 pg. DNAP the average DNAP content per hepatocyte nucleus should be

$$\frac{(0.65 \times 12.28)+(2 \times 0.65 \times 79.18)+(4 \times 0.65 \times 8.14)+(8 \times 0.65 \times 0.4)}{100}$$

$$= 1.341 \text{ pg.}$$

But the hepatocytes account for only 60% of the nuclei of the liver (Abercrombie & Harkness, 1951). Assuming that the remainder are all diploid the average DNAP content per nucleus for the whole liver should be

$$\frac{(1.341 \times 60) + (0.65 \times 40)}{100}$$

$$= 1.06 \text{ pg.}$$

This figure also is in reasonable agreement with the value of 0.913 pg. found experimentally for isolated liver nuclei.

The lower figures found for average DNAP per nucleus in the livers of young animals (Table 21) may also be explained in terms of polyploidy since Swift (1950a) has

shown that in mouse liver the proportion of Class II and Class III nuclei is lower in young animals than in adults. Moreover, McKellar (1949) has found, using Jacobj's (1925) histological method, that the proportion of polyploid hepatocyte nuclei in the liver of the rat increases with age. For example, in a young rat (body weight 37 g.) only 23% of the hepatocyte nuclei were tetraploid as against 70% in an adult animal (body weight 245 g.). (There is no mention of the occurrence of octoploid or 16-ploid nuclei.) Assuming again that the diploid nucleus contains 0.65 pg. DNAP this means that the average DNAP content of the hepatocyte nuclei should be

$$\frac{(0.65 \times 77) + (2 \times 0.65 \times 23)}{100} = 0.799 \text{ pg.}$$

for the young animal and

$$\frac{(0.65 \times 30) + (2 \times 0.65 \times 70)}{100} = 1.105 \text{ pg.}$$

for the adult. Assuming also that the hepatocytes in each case accounted for 60% of the total population of nuclei in the liver and that the non-hepalocyte nuclei were all diploid, the average DNAP content of all the liver nuclei should be

$$\frac{(0.799 \times 60) + (0.65 \times 40)}{100} = 0.740 \text{ pg.}$$

for the young animal and

$$\frac{(1.105 \times 60) + (0.65 \times 40)}{100} = 0.924 \text{ pg.}$$

for the adult. These figures also are in good agreement with the values of 0.758 pg. and 0.913 pg. found experimentally for nuclei isolated from the livers of young and adult animals respectively (Table 20).

It seems therefore reasonable to conclude (a) that the high average DNAP content per nucleus in rat liver as compared with other rat tissues is due to the occurrence of polyploidy, and (b) that the nuclei isolated from rat liver by the technique used in the present series of experiments constitute a reasonably representative sample of the whole population of nuclei in this organ.

As has already been pointed out in Section 2.1, the results obtained for liver regenerating after partial hepatectomy are of particular interest because of the extremely high rate of growth in this tissue (see Table 25). While mitosis occurs with a frequency of 1 in 7000 to 1 in 20,000 in the liver of a normal adult rat (Brues & Marble, 1937; Marshak & Byron, 1945) the frequency may reach 1 in 40 for parenchymal cells and 1 in 100 for bile duct cells during the four days following partial hepatectomy (Abercrombie & Harkness, 1951). It is obvious that DNA must be synthesized during cell multiplication but there have been conflicting reports on the relation of DNA synthesis to the stages of the mitotic cycle. Swift (1950a) and Walker & Yates (1952a, b) have concluded, on the basis of cyto-

photometric experiments, that DNA synthesis precedes mitosis so that nuclei about to undergo mitosis contain a double quantity of DNA. This view is, to some extent, supported by the autoradiograph experiments of Howard & Pelc (Howard & Pelc, 1951; Pelc & Howard, 1952) which appear to show that in the growing bean root uptake of radioactive phosphorus into DNA occurs during interphase. If it is correct a rapidly growing tissue containing many nuclei which are about to divide should have an increased average DNA content per nucleus. On the other hand, Seshachar (1950), Pasteels & Lison (1950b, c) and Marinone (1951) have all concluded that half the normal nuclear content of DNA goes to each daughter nucleus at metaphase and that the full nuclear content is restored by synthesis of new DNA at telophase. This process would not be expected to affect the average DNA content per nucleus in the tissue unless the nuclear membranes of the daughter nuclei formed before DNA synthesis was complete, in which case the average DNA content per nucleus would be depressed. The fact that, in the present investigation, the average DNA content per nucleus in the liver during the first four days of regeneration after partial hepatectomy (Table 25) was 10 - 50% above the mean value found for unoperated animals of the same sex, strain and body weight (Table 20 and Table 21, Section1)

would appear to support Swift's hypothesis that DNA synthesis precedes mitosis.

It is of some interest that even 6 - 10 days after the operation, when the phase of rapid growth had ended, the average DNAP content per nucleus (Table 25) was still above the normal level for unoperated animals. The same trend is apparent in the results of Price & Laird (1950). This suggests that the restored liver may perhaps contain an increased proportion of polyploid nuclei. Sulkin (1943) has, in fact, shown by measurements of nuclear volume that in restored liver (28 days after partial hepatectomy) 5.70% of the hepatocyte nuclei are diploid, 68.10% tetraploid, 22.40% octoploid and 3.80% 16-ploid. On the assumption that the diploid nucleus contains 0.65 pg. DNAP the average DNAP content per hepatocyte nucleus in such a liver should be

$$\frac{(0.65 \times 5.70)+(2 \times 0.65 \times 68.10)+(4 \times 0.65 \times 22.40)+(8 \times 0.65 \times 3.80)}{100}$$

$$= 1.703 \text{ pg.}$$

Again assuming that the hepatocytes account for only 60 % of the nuclei in the liver and that the non-hepatocyte nuclei are all diploid, it follows that the average DNAP content per nucleus for the whole liver should be

$$\frac{(1.703 \times 60) + (0.65 \times 40)}{100}$$

$$= 1.28 \text{ pg.}$$

The difference between this figure and that of 1.06 pg. calculated above from Sulkin's results for normal liver is of the same order as that found in the present series of experiments between the average DNAP content of nuclei isolated from restored liver 6 - 10 days after hepatectomy (Table 25), and the average DNAP content of nuclei isolated from the livers of normal adult animals (Table 20 and Table 21, Section 1). It seems, therefore, that this latter difference may be completely explained in terms of a change in the proportions of polyploid nuclei. There is a parallel here with the difference between the livers of young and adult animals. In both cases growth of the liver is accompanied by an increase in the proportion of polyploid nuclei and a consequent increase in the average DNA content per nucleus.

It appears possible, therefore, that in the resting nuclei of rat tissues each set of chromosomes may contain a constant amount of DNA. If this is the case it follows that in adult tissues in which mitoses are infrequent the average DNA content per nucleus should not be affected by any circumstance or treatment which does not lead to either multiplication or destruction of the nuclei. The results obtained for the livers of adult rats shown in Table 21, Section 2 support this conclusion. None of the dietary conditions affected the average DNA content of the nuclei

although some of them produced marked changes in the chemical composition or histological appearance of the liver. Similar results for the effect of fasting have been reported by Mirsky & Ris (1951), McIndoe & Davidson (1952) and Fukuda & Sibatani (1953), for the effect of protein deficiency by Campbell & Kosterlitz (1952) and Villela (1952), and for the effect of vitamin B_{12} deficiency by Rose & Schweigert (1952).

There is some evidence to suggest that the response of young animals to protein deficiency may differ from that of adults. Ely & Ross (1951a, b) have reported that in rats weighing 130 - 150 g. the average DNA content per nucleus in the liver, pancreas and small intestine is higher in animals maintained on a protein-free diet than in animals maintained on a complete diet. Since this difference can be demonstrated both by chemical analysis of counted suspensions of isolated nuclei and by cytophotometric measurements on individual nuclei in tissue sections it is presumably not due to a difference in the proportion of polyploid nuclei. This conclusion is supported by the work of Lecomte & Smul (1952) who found by cytophotometric measurements on individual nuclei that in rats weighing 60 - 65 g. protein deficiency for 28 days resulted in an increase in the average DNA content of the Class II (i.e., tetraploid) liver nuclei. It is not yet clear what sig-

nificance may be attached to these observations. It may be assumed that many, if not most, of the cells in the livers of young animals will eventually undergo mitosis and that before doing so they will double their content of DNA. It is conceivable that protein deficiency might inhibit mitosis without greatly affecting the premitotic synthesis of DNA. The consequent increase in the proportion of nuclei ready to undergo mitosis and containing twice the normal content of DNA would tend to raise the average DNA content per nucleus for the tissue. This hypothesis finds some support in the observation of Ely & Ross (1952) that the number of cells per liver in young rats kept on a protein-free diet for 20 or 30 days is only 75% of that found in control animals on a complete diet. Nevertheless, the question clearly requires further study, particularly as Fukuda & Sibatani (1953) have found that maintaining young rats on a very low calorie intake for 4 weeks so that growth is almost completely inhibited does not affect the average DNA content of the liver nuclei.

The results for the composition of whole liver of adult rats shown in Table 22, Sections 3, 4 and 5 confirm and extend the observations previously made by other workers on the remarkable constancy of the total DNA content of the liver, i.e., of the total number of nuclei which it contains (Davidson, 1947a, 1953; Campbell & Kosterlitz, 1950;

Mandel, 1951). In this organ the nuclei and their characteristic chemical component, DNA, form a constant element while LP, PN and cell mass (in short, the cytoplasm) vary a great deal depending on the nutritional status of the animal. RNA follows an intermediate pattern; in the initial stages of conditions like starvation or protein deficiency there is a marked decrease in the RNA content of the liver but this trend is not progressive like the changes in the constituents mentioned above. A similar trend can be seen in the data of Kosterlitz (1947). These observations suggest that only part of the RNA of the cell is labile. This conclusion is also supported by the data obtained by Muntwyler and his associates in the course of tissue fractionation experiments on rat liver. These indicate that protein deficiency affects the RNA content of the so-called supernatant or cell sap fraction (see Section 1.4) much less than that of the other tissue fractions (Muntwyler, Seifter & Harkness, 1950; Seifter, Muntwyler & Harkness, 1950). The effect is more clearly seen if Muntwyler's figures, which are presented as amounts per unit weight of liver, are recalculated as total amounts per liver. Wikramanayake, Heagy & Munro (1952) have also observed that protein deficiency has a selective effect on the RNA content of different cellular fractions of the rat liver.

As has already been pointed out (Section 2.1), an important consequence of the constancy, at least under certain conditions, of the average DNA content per nucleus is that the amount of DNA present in a tissue can be used to estimate the number of nuclei which it contains. Several groups of workers have already described methods for estimating the number of nuclei in a tissue. Brues, Drury & Brues (1936) have counted the number of heptatocyte nuclei in a known area of a liver section of known thickness and hence calculated the number of such nuclei in the whole liver. The accuracy of this method is very doubtful since it depends entirely on the thickness of the section used for counting being exactly known. It is, moreover, too tedious and time-consuming for routine use. This second criticism applies also to the very complex histological method recently devised by Carnes, Weissman & Goldberg (1952) which involves measurements of the relative volumes of nuclei and cytoplasm, of the average absolute volume of the nuclei, and of the total volume of the liver. These methods are applicable, of course, to all types of tissue. It has already been indicated above that the number of nuclei in a sample of liver tissue may be estimated by homogenizing in dilute citric acid and counting the nuclei in the homogenate (Price & Laird, 1950). Mizen & Petermann (1952) have recently employed a modification of this

method in which 0.88M sucrose containing a trace of calcium chloride is used in place of dilute citric acid, to determine the number of nuclei in normal and leukaemic mouse spleen. These methods are comparatively simple and rapid but they can only be applied to soft tissues with a dense nuclear population, like liver and spleen. Moreover, it is always possible that fallaciously low results may be obtained due to destruction of nuclei during homogenization. It seems therefore that the most reliable, as well as the most general, method of estimating the number of nuclei in a tissue is to divide its total content of DNA by its average DNA content per nucleus (as determined by analysis of isolated nuclei).

A second consequence of the constancy of the average DNA content per nucleus is that, as has already been observed (Section 2.1), it makes possible the calculation of the average composition per cell of a sample of tissue. In tissue analysis the results obtained must necessarily be related to some standard of reference - usually the fresh weight or dry weight of the tissue. This means that an apparent change in a measured variable may sometimes be due to a change in the standard of reference. For example, in the present investigation it was found that during a 48-hour fast the concentrations of DNAP, LP and RNAP per 100 g. of fresh liver all increased (Table 22, Section 4).

But calculations of the total amounts per liver show that the DNAP content remained unchanged while the LP, PN and RNAP actually decreased by 20 - 30%. The apparent increase, on a fresh weight basis, was due to a fall of almost 40% in the liver weight and could be quite misleading. Obviously, it is desirable that the standard of reference should not change during an experiment.

The simplest way of avoiding this difficulty is to quote the total amount of each component per liver as is done in the example above. This method has two limitations: (1) it is only applicable where a whole organ is available for analysis and cannot be used for tissues such as bone marrow or for biopsy specimens; and (2) changes in cell number cannot be distinguished from changes in average cell composition. A more satisfactory procedure is to use the DNA content of the tissue as a measure of cell number and the ratios of other tissue components to DNA as a measure of average cell composition. This overcomes the limitations of the previous method and has the additional advantage that it may bring to light significant changes in cell composition which would otherwise be obscured by random variations in cell number. For example, the changes in liver weight and total content of LP and RNAP during the second week on the protein-free diet (Table 21, Section 4) are not statistically significant, while the change in total PN content is

significant only on the 5% level (see Table 62). If the results are referred to DNAP, however, it becomes apparent that there are significant decreases in average cell mass ($P < 0.001$) and average cell content of LP ($P < 0.01$) and PN ($P < 0.02$) while the average content of RNAP per cell has not changed (see Table 57).

It should be mentioned that Harrison (1953b) has recently attempted to use this approach to calculate not only the average composition of all the cells in the rat liver but also the average composition of the diploid cells alone. Since, however, her calculations are based on the false assumption that the liver cells are all of the same type (viz., hepatocytes of varying degrees of ploidy) the results which she has obtained in this way are of rather dubious significance.

The analysis of a tissue which is being infiltrated with tumour cells is a case which calls for special consideration. The composition of such a tissue may be affected by at least three distinct factors.

(i) As the number of tumour cells increases the composition as a whole will tend to approach that of the tumour cells. If the tumour nuclei are all diploid while some of the nuclei of the normal tissue are polyploid, as in rat liver, the progress

of the tumour will be marked by a steady decrease in the average DNA content per nucleus. If, on the other hand, the tumour nuclei contain more DNA than the nuclei of the normal tissue, as is the case in certain mouse tumours (Klein, Kurnick & Klein, 1950; Goldberg, Klein & Klein, 1950; Klein, 1951; Klein & Klein, 1952; Leuchtenberger, Klein & Klein, 1952a, b; Petermann & Schneider, 1951; Bader, 1953) and in at least one fowl tumour (McIndoe & Davidson, 1952), there will be a steady increase in the average DNA content per nucleus.
(ii) If the tumour is growing rapidly it may contain a significant proportion of nuclei which are about to divide and have for that reason more than their normal resting complement of DNA. This would tend to raise the average DNA content per nucleus.
(iii) If the growing nodules of tumour tissue compress the normal tissue to such an extent that it degenerates, the nuclei of the latter may undergo pyknosis and lose DNA (Leuchtenberger, 1950).

Since each of these factors has some effect on the average DNA content per nucleus it might be thought that little purpose would be served by referring the analyses of such tissues to DNA. Inspection of the results obtained in the present investigation with rats fed a

carcinogenic diet (Table 24) shows, however, that this is not the case. In rats nos.H1, H7, H8, H9, H10, H11, 465, 466 and 467 infiltration of the liver with nodules of tumour tissue had proceeded to the point where it was clearly visible to the naked eye. Yet the liver weights of these animals and the total contents per liver of LP, PN and RNAP are not strikingly different from those found in the controls. Nor has the growth of the tumour nodules greatly affected the concentrations of LP, PN and RNAP per 100 g. fresh tissue. It is only when the total amounts of DNAP per liver are examined that it becomes clear that there has been an increase in the total number of sets of chromosomes per liver of anything between 25% and 100%. This might be due to an increase in the total number of nuclei in the liver, or in the proportion of polyploid nuclei, or both. Since in the present case there is a simultaneous fall in the average DNAP content per nucleus the first explanation is obviously the correct one. Thus it is clear that the interpretation of the analysis of a tumour-bearing tissue is greatly facilitated if its total content of DNA, and preferably also its average DNA content per nucleus, are known.

2.5 Summary and conclusions.

The general conclusions which emerge from the results obtained in the present series of experiments on the DNA content of rat cell nuclei may be summarized as follows.

In both young and adult animals, spleen, lung, small intestine, salivary gland, leucocytes, heart, bone marrow, pancreas and thymus all have the same average DNA content per nucleus. The average DNA content per nucleus in the liver of the adult animal is 30% greater than the figure obtained for the non-hepatic tissues and is increased after partial hepatectomy. The corresponding figures for young animals and embryos are much lower and closer to those found for the non-hepatic tissues.

These observations are clearly inconsistent with the Boivin-Vendrely hypothesis as originally enunciated, viz., that in a given species all the somatic nuclei have the same content of DNA. They are, however, quite consistent with the assumption that in any given species the DNA content per set of chromosomes is constant for all resting interphase nuclei, since there is evidence of a purely histological nature that rat liver contains a considerable proportion of polyploid nuclei which is greater in the adult than in the young animal and which is increased after partial hepatectomy. This assumption might conveniently be termed the modified Boivin-Vendrely hypothesis.

If the DNA content per set of chromosomes is, in fact, constant for all the resting nuclei of a given species mitosis must be accompanied by a sudden synthesis of DNA. Since it has been found in the present series of experiments that the average DNA content per nucleus in the liver is markedly increased during the phase of rapid compensatory hyperplasia which takes place during the first four days following partial hepatectomy it is probable that this synthesis occurs, at least to some extent, in prophase or late interphase.

If the DNA content per set of chromosomes is constant it follows also that, even in a heteroploid tissue such as rat liver, the average DNA content per nucleus should not be affected by treatments which do not lead to multiplication or destruction of the nuclei. In accordance with this prediction it has been found that short-term dietary treatments do not affect the average DNA content per nucleus in the rat liver.

It seems, therefore, quite probable that in the rat the DNA content per set of chromosomes may be constant for all resting interphase nuclei.

Since the average DNA content per nucleus is also constant within limits the DNA content of a tissue may be taken as a measure of the number of nuclei which it contains

and the ratios of other tissue components to DNA as a measure of average cell composition. It has been shown above that this method of expressing the results of tissue analysis has considerable advantages over more conventional methods, particularly in the case of tumour-bearing tissues.

2.6 Statistical analyses of data presented in Tables 20, 21 and 22.

Table 28.

Statistical analysis of data presented in Table 20. Analysis of variance to determine significance of differences in mean values of DNAP content per nucleus, as determined by phosphorus estimation, for kidney, spleen, lung, small intestine, salivary gland, heart, bone marrow and pancreas, of adult animals.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	Variance ratio (F)
Tissues	27	0.135731	0.004307	0.856
Residual	8	0.034456	0.005027	
Total	35	0.170187		

For $n_1 = 30$, $n_2 = 8$, $F = 3.08$ at the 5% significance level. There is therefore no significant difference between the means for the different tissues.

Table 29.

Statistical analysis of data presented in Table 20. Analysis of variance to determine significance of differences in mean values of DNAP content per nucleus, as determined by deoxypentose estimation, for kidney, spleen, lung and small intestine, of adult animals.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio (F)
Tissues	3	0.040263	0.013421	1.481
Residual	20	0.181296	0.009065	
Total	23	0.221559		

For $n_1 = 3$, $n_2 = 20$, $F = 3.10$ at the 5% significance level.

There is therefore no significant difference between the means for the different tissues.

Table 30.

Statistical analysis of data presented in Table 20.

Analysis of variance to determine significance of differences in mean values of DNAP content per nucleus, as determined by ultraviolet absorption measurements, for kidney, spleen, lung, small intestine, salivary gland, leucocytes, heart and pancreas, of adult animals.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio (F)
Tissues	7	0.018669	0.002667	0.729
Residual	24	0.087837	0.003660	
Total	31	0.106506		

For $n_1 = 7$, $n_2 = 24$ $F = 3.50$ at 5% significance level.

There is therefore no significant difference between the means for the different tissues.

Table 31.

Statistical analysis of data presented in Table 20. Analysis of variance to determine significance of differences in mean values of DNAP content per nucleus, as determined by phosphorus estimation, for kidney, spleen, lung, small intestine, salivary gland and thymus, of young animals.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio (F)
Tissues	5	0.019317	0.003863	2.47
Residual	7	0.010924	0.001561	
Total	12	0.030241		

For $n_1 = 5$, $n_2 = 7$ $F = 3.97$ at the 5% significance level.

There is therefore no significant difference between the means for the different tissues.

Table 32.

Statistical analysis of data presented in Table 20.

Analysis of variance to determine significance of differences in mean values of DNAP content per nucleus, as determined by deoxypentose estimation, for kidney, spleen and thymus, of young animals.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio (F)
Tissues	2	0.000621	0.000311	0.0552
Residual	5	0.028195	0.005639	
Total	7	0.028816		

For $n_1 = 2$, $n_2 = 5$, $F = 13.27$ at 5% significance level.

There is therefore no significant difference between the means for the different tissues.

Table 33.

Statistical analysis of data presented in Table 20. Analysis of variance to determine significance of differences in mean values of DNAP content per nucleus, as determined by ultraviolet absorption measurements, for kidney, spleen, lung, small intestine, salivary gland and thymus, of young animals.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio (F)
Tissues	5	0.013230	0.002646	0.646
Residual	7	0.028769	0.004110	
Total	12	0.041999		

For $n_1 = 5$, $n_2 = 7$, $F = 3.97$ at the 5% significance level.

There is therefore no significant difference between the means for the different tissues.

Table 34.

Statistical analysis of data presented in Table 20. "t" tests to determine significance of differences in average DNAP content per nucleus as determined by phosphorus estimation, between the tissues of the adult male albino rat.

Tissues compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
Liver and kidney	44	9.74	<0.001
" " spleen	44	10.40	<0.001
" " lung	42	8.34	<0.001
" " small intestine	40	4.84	<0.001
" " salivary gland	38	3.54	<0.01
" " leucocytes	40	7.03	<0.001
" " pancreas	38	3.97	<0.001

Table 35.

Statistical analysis of data presented in Table 20.

"t" tests to determine significance of differences in mean values for the average DNAP content per nucleus, as determined by deoxypentose estimation, between different tissues of the adult male albino rat.

Tissues compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
Liver and kidney	35	6.31	< 0.001
" " spleen	36	6.12	< 0.001
" " lung	33	4.79	< 0.001
" " small intestime	32	2.87	< 0.01

Table 36.

Statistical analysis of data presented in Table 20. "t" tests to determine significance of differences in mean values for average DNAP content per nucleus, as determined by ultraviolet absorption measurements, between different tissues of the adult male albino rat.

Tissues compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
Liver and kidney	44	5.89	< 0.001
" " spleen	44	6.44	< 0.001
" " lung	42	5.41	< 0.001
" " small intestine	40	4.77	< 0.001
" " salivary gland	38	2.99	< 0.01
" " pancreas	38	2.08	< 0.05

Table 37.

Statistical analysis of data presented in Table 20. "t" tests to determine significance of differences in mean values for average DNAP content per nucleus in the liver between young and adult rats.

Method of determination	Degrees of freedom	Value of "t" found	Corresponding value of "P"
Phosphorus estimation	49	6.76	< 0.001
Deoxypentose estimation	39	3.65	< 0.001
Ultraviolet absorption measurements	49	3.91	< 0.001

Table 38.

Statistical analysis of data presented in Table 20.

"t" tests to determine significance of differences in mean values for average DNAP content per nucleus in the liver between adult and embryo rats.

Method of determination	Degrees of freedom	Value of "t" found	Corresponding value of "P"
Phosphorus estimation	40	3.20	< 0.01
Deoxypentose estimation	32	1.46	> 0.1
Ultraviolet absorption measurements	40	2.78	< 0.01

Table 39.

Statistical analysis of data presented in Table 20. "t" tests to determine significance of differences in mean values for average DNAP content per nucleus in the liver between young rats and embryo rats.

Method of determination	Degrees of freedom	Value of "t" found	Corresponding value of "P"
Phosphorus estimation	15	0.592	> 0.5
Deoxypentose estimation	13	0.684	> 0.5
Ultraviolet absorption measurements	15	0.792	> 0.4

Table 40.

Statistical analysis of data presented in Table 21, Section 1.

Analysis of variance to determine significance of differences in mean values of DNAP content per nucleus in the liver, as determined by phosphorus estimation, between different groups of adult animals on stock diet.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio (F)
Groups	6	0.039927	0.006655	0.772
Residual	102	0.879213	0.008620	
Total	108	0.919140		

For $n_1 = 6$, $n_2 = 100$, $F = 2.19$ at the 5% significance level.

There is therefore no significant difference between the means for the different groups.

Table 41.

Statistical analysis of data presented in Table 21, Section 1.

Analysis of variance to determine significance of differences in mean values of DNAP content per nucleus in the liver, as determined by deoxypentose estimation, between different groups of adult animals on stock diet.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio (F)
Groups	6	0.112680	0.01878	1.18
Residual	87	1.378729	0.015847	
Total	93	1.491409		

For $n_1 = 6$, $n_2 = 80$ $F = 2.21$ at the 5% significance level.

There is therefore no significant difference between the means for the different groups.

Table 42.

Statistical analysis of data presented in Table 21, Section 1.

Analysis of variance to determine significance of differences in mean values for DNAP content per nucleus in the liver as determined by ultraviolet absorption measurements, between different groups of adult animals on stock diet.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio (F)
Groups	6	0.049962	0.008327	0.664
Residual	100	1.254355	0.01254	
Total	106	1.304317		

For $n_1 = 6$, $n_2 = 100$ $F = 2.19$ at the 5% significance level.

There is therefore no significant difference between the means for the different groups.

Table 43.

Statistical analysis of data presented in Table 21, Section 2.

Analysis of variance to determine significance of differences in mean values of DNAP content per nucleus in the liver, as determined by phosphorus estimation, between groups of adult male albino rats on various diets.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio (F)
Groups	9	0.038752	0.004306	0.550
Residual	109	0.853593	0.007831	
Total	118	0.892345		

For $n_1 = 9$, $n_2 = 100$ $F = 1.97$ at the 5% significance level.

There is therefore no significant difference between the means for the different groups.

Table 44.

Statistical analysis of data presented in Table 21, Section 2.

Analysis of variance to determine significance of differences in mean values for DNAP content per nucleus in the liver, as determined by deoxypentose estimation, between groups of adult male albino rats on various diets.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio (F)
Groups	9	0.069743	0.007763	0.589
Residual	82	1.081114	0.013184	
Total	93	1.150857		

For $n_1 = 9$, $n_2 = 80$ $F = 1.99$ at the 5% significance level.

There is therefore no significant difference between the means for the different groups.

Table 45.

Statistical analysis of data presented in Table 21, Section 2.

Analysis of variance to determine significance of differences in mean DNAP content per nucleus in the liver, as determined by ultraviolet absorption measurements, between groups of adult male albino rats on various diets.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio (F)
Groups	9	0.037453	0.004364	0.4259
Residual	107	1.096354	0.010246	
Total	116	1.133806		

For $n_1 = 9$, $n_2 = 100$ $F = 1.97$ at the 5% significance level.

There is therefore no significant difference between the means for the different groups.

Table 46.

Statistical analysis of data presented in Table 21, Section 3.

"t" tests to determine significance of differences in mean values of DNAP content per nucleus between alloxan-diabetic rats and controls.

Method of determination	Degrees of freedom	Value of "t" found	Value of "t" required for significance at 5% level
Phosphorus estimation	7	0.56	2.365
Deoxypentose estimation	5	1.76	2.571
Ultraviolet absorption	7	0.27	2.365

There is therefore no significant difference between the means for the two groups of animals, no matter which of the three methods of estimating DNAP is used.

Table 47.

Statistical analysis of data presented in Table 22, Section 1.

"t" tests to determine significance of differences in mean values for the composition of whole liver tissue between male albino rats of body weight 212±8 g. and female albino rats of body weight 207±8g.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
Concentration of DNAP (mg./100 g.liver)	29	4.96	< 0.001
Total amount of LP (mg./liver)	29	4.21	< 0.001
Total amount of PN (mg./liver)	15	1.61	> 0.1
Total amount of RNAP (mg./liver)	29	1.35	> 0.1
Total amount of DNAP (mg./liver)	29	1.31	> 0.2

Table 48.

Statistical analysis of data presented in Table 22, Section 1.

"t" tests to determine significance of differences in mean values for the composition of whole liver tissue between male hooded rats of body weight 215±12 g. and female hooded rats of body weight 215±13 g.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
Concentration of DNAP (mg./100 g. liver)	20	3.14	< 0.01
Total amount of LP (mg./liver)	20	4.21	< 0.001
Total amount of PN (mg./liver)	20	0.92	> 0.3
Total amount of RNAP (mg./liver)	20	0.68	> 0.5
Total amount of DNAP (mg./liver)	20	2.26	< 0.05

Table 49.

Statistical analysis of data presented in Table 22, Section 1.

"t" tests to determine significance of differences in mean values for the composition of whole liver tissue between male albino rats of body weight 212±8 g. and female albino rats of body weight 207±8 g.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
LP (pg./pg. DNAP)	29	3.48	< 0.01
PN (pg./pg. DNAP)	15	2.57	< 0.05
RNAP (pg./pg. DNAP)	29	3.46	< 0.01
Tissue mass (pg./pg. DNAP)	29	4.71	< 0.001

Table 50.

Statistical analysis of data presented in Table 22, Section 1.

"t" tests to determine significance of differences in mean values for the composition of whole liver tissue between male hooded rats of body weight 215±12 g. and female hooded rats of body weight 215±13 g.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
LP (pg./pg. DNAP)	20	4.37	< 0.001
PN (pg./pg. DNAP)	20	3.13	< 0.01
RNAP (pg./pg. DNAP)	20	3.15	< 0.01
Tissue mass (pg./pg. DNAP)	20	3.12	< 0.01

Table 51.

Statistical analysis of data presented in Table 22, Section 2.

"t" tests to determine significance of differences in mean values for the composition of whole liver tissue between non-pregnant female albino rats of body weight 199±11 g. and pregnant female albino rats of body weight 234±17 g.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
Total amount of DNAP (mg./liver)	22	0.69	> 0.4
LP (pg./pg. DNAP)	22	3.98	< 0.001
PN (pg./pg. DNAP)	14	2.00	> 0.05
RNAP (pg./pg. DNAP)	22	5.05	< 0.001
Tissue mass (pg./pg. DNAP)	22	2.55	< 0.02

Table 52.

Statistical analysis of data presented in Table 22, Sections 3 and 4.

Analysis of variance to determine significance of differences in mean values of total DNAP content per liver between groups of adult male albino rats on various diets.

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Variance ratio (F)
Groups	9	0.7239	0.0804	1.05
Residual	123	9.4050	0.0765	
Total	132	10.1289		

For $n_1 = 9$, $n_2 = 125$ $F = 1.95$ at the 5% significance level.

There is therefore no significant difference between the means for the different groups.

Table 53.

Statistical analysis of data presented in Table 22, Section 2.

"t" tests to determine significance of differences in mean values for the composition of whole liver tissue between male albino rats of body weight 221±14 g. on stock diet and similar rats (of initial body weight 224±12), after a 72-hour fast.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
LP (pg./pg. DNAP)	46	4.06	<0.001
PN (pg./pg. DNAP)	27	2.24	<0.05
RNAP (pg./pg. DNAP)	46	4.31	<0.001
Tissue mass (pg./pg. DNAP)	46	6.94	<0.001

Table 54.

Statistical analysis of data presented in Table 22, Section 3.

"t" tests to determine significance of differences in mean values for composition of whole liver tissue between male albino rats on semi-synthetic diet (b) and similar animals after a 48-hour fast.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
LP (pg./pg. DNAP)	26	5.52	< 0.001
PN (pg./pg. DNAP)	15	4.25	< 0.001
RNAP (pg./pg. DNAP)	26	10.28	< 0.001
Tissue mass (pg./pg. DNAP)	26	9.38	< 0.001

Table 55.

Statistical analysis of data presented in Table 22, Section 4.

"t" tests to determine significance of differences in mean values for the composition of whole liver between male albino rats on the semi-synthetic diet and similar animals on the thiamine-deficient diet.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
LP (pg./pg. DNAP)	23	6.18	< 0.001
PN (pg./pg. DNAP)	12	2.72	< 0.02
RNAP (pg./pg. DNAP)	23	9.89	< 0.001
Tissue mass (pg./pg. DNAP)	23	8.29	< 0.001

Table 56.

Statistical analysis of data presented in Table 22, Section 4.

"t" tests to determine significance of differences in mean values for composition of whole liver tissue between male albino rats on the semisynthetic diet and similar animals maintained on the protein free diet for 7 days.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
LP (pg./pg. DNAP)	25	8.15	< 0.001
PN (pg./pg. DNAP)	15	5.28	< 0.001
RNAP (pg./pg. DNAP)	25	11.08	< 0.001
Tissue mass (pg./pg. DNAP)	25	4.37	< 0.001

Table 57.

Statistical analysis of data presented in Table 22, Section 4.

"t" tests to determine significance of differences in mean values for composition of whole liver tissue between male albino rats maintained on the protein free diet (c) for 7 days and similar animals maintained on the same diet for 15 days.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
LP (pg./pg. DNAP)	22	3.16	< 0.01
PN (pg./pg. DNAP)	14	2.91	< 0.02
RNAP (pg./pg. DNAP)	22	0.40	> 0.5
Tissue mass (pg./pg. DNAP)	22	3.82	< 0.001

Table 58.

Statistical analysis of data presented in Table 22, Section 3.

"t" tests to determine significance of differences in mean values for composition of whole liver tissue between male albino rats on the stock diet and similar animals maintained on the high-fat diet for 14 days.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
LP (pg./pg. DNAP)	56	6.11	< 0.001
PN (pg./pg. DNAP)	32	2.69	< 0.02
RNAP (pg./pg. DNAP)	56	6.09	< 0.001
Tissue mass (pg./pg. DNAP)	56	1.60	> 0.10

Table 59.

Statistical analysis of data presented in Table 22, Section 4.

"t" tests to determine significance of differences in mean values for composition of whole liver tissue between male albino rats on the semi-synthetic diet and similar animals maintained on the thioacetamide-containing diet for 7 days.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
LP (pg./pg. DNAP)	16	0.34	>0.5
PN (pg./pg. DNAP)	9	1.51	<0.2
RNAP (pg./pg. DNAP)	16	1.97	<0.1
Tissue mass (pg./pg. DNAP)	16	0.95	>0.5

Table 60.

Statistical analysis of data presented in Table 22, Sections 3 and 4.

"t" test to determine significance of difference in mean values for composition of whole liver tissue between male albino rats of body weight 221±14 g. on stock diet and similar rats of body weight 223±12 g. on semi-synthetic diet.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
LP (pg./pg. DNAP)	54	2.68	< 0.01

Table 61.

Statistical analysis of data presented in Table 22, Section 5.

"t" tests to determine significance of differences in mean values for the composition of whole liver tissue between rats made diabetic by injection of alloxan and control animals injected with saline.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
Liver weight	7	3.78	< 0.01
Total amount of LP (mg./liver)	7	3.50	< 0.01
Total amount of RNAP (mg./liver)	7	3.00	< 0.02
Total amount of DNAP (mg./liver)	7	1.83	> 0.1
LP (pg./pg. DNAP)	7	0.62	> 0.5
RNAP (pg./pg. DNAP)	7	1.26	> 0.2
Tissue mass (pg./pg. DNAP)	7	1.86	> 0.1

Table 62.

Statistical analysis of data presented in Table 22, Section 4.

"t" tests to determine significance of differences in mean values for the composition of whole liver tissue between male albino rats maintained on the protein-free diet (c) for 7 days and similar animals maintained on the same diet for 15 days.

Means compared	Degrees of freedom	Value of "t" found	Corresponding value of "P"
Liver weight	23	1.61	> 0.1
Total amount of LP (mg./liver)	22	0.76	> 0.4
Total amount of PN (mg./liver)	14	2.34	< 0.05
Total amount of RNAP (mg./liver)	22	0.41	> 0.5

Part III.

The Relative Deoxyribonucleic Acid Content of Individual Nuclei in Various Rat Tissues as Determined by Cytophotometry.

The Relative Deoxyribonucleic Acid Content of Individual Nuclei in Various Rat Tissues as Determined by Cytophotometry.

3.1 Objects of the cytophotometric investigation.

When the series of experiments described in the preceding pages was concluded the question arose whether it ought to be supplemented by a cytophotometric investigation. Five considerations appeared to make this course of action advisable.

1) One of the main pieces of evidence on which Boivin, Vendrely & Vendrely (1948) based their identification of genes as macromolecules of DNA was their observation that in certain species (bull, carp, pike) the average DNA content of the spermatozoa was almost exactly half that found for the somatic nuclei (Vendrely & Vendrely, 1948; Vendrely, 1952). Mirsky & Ris (1949), as has already been indicated (Section 1.11), were able to confirm this finding in the case of certain fish and amphibians. They reported, however, that bull spermatozoa contained significantly less than half the DNA content of nuclei isolated from calf or beef tissues. Under these circumstances it was clearly desirable that in the present investigation the DNA content of rat spermatozoa should be determined for comparison with the figures found for the somatic nuclei of the same species. Unfortunately, this proved impracticable as the number of

spermatozoa which could be obtained post mortem by washing out the vas deferens with physiological saline was too small for chemical analysis and attempts to obtain semen from living animals by inducing ejaculation by electrical stimulation under ether anaesthesia were completely unsuccessful. On the other hand it was apparent that no difficulty would be experienced in obtaining the very small number of spermatozoa required for cytophotometric estimation.

2) In 1950 Swift (Swift, 1950a) published an account of an extensive survey by the cytophotometric method of the DNA content of nuclei in a variety of mouse tissues. His results indicated that in this species Class I nuclei, containing the diploid amount of DNA, occurred in all the tissues examined and that in most tissues all the nuclei were of this type. Class II and Class III nuclei, containing respectively twice and four times the diploid amount of DNA, were also found in some tissues, notably liver and pancreas. Examination of frog tissues, on the other hand, seemed to show that in this species all somatic nuclei contained the same amount of DNA with the exception of a few liver nuclei which contained twice this amount. Quite apart from the support which these observations lend to the theory that in any given species the DNA content per

set of chromosomes is constant, they are of interest inasmuch as they indicate that in rodents polyploidy may not be confined to the liver. It might be thought that the results of the present investigation, indicating as they do no significant differences in average DNA content per nucleus between kidney, spleen, lung, small intestine, salivary gland, leucocytes, heart, bone marrow, pancreas and thymus in the rat, exclude the possibility of polyploidy in any of these tissues, but this is not necessarily the case. If, for example, 5% of the nuclei in pancreas are tetraploid and the rest diploid, while all those in kidney are diploid the difference in average DNA content per nucleus between the two tissues will only be 5%, which may well be too small to be readily detected by chemical analysis of counted suspensions of isolated nuclei. Such a small proportion of polyploid nuclei, which might be of considerable biological importance, could be demonstrated with ease and certainty by the cytophotometric technique.

3) The only cytophotometric investigation of rat tissues comparable with Swift's work on the mouse is that carried out by Pasteels & Lison (1950a, c). In agreement with Swift these workers found that in some of the tissues they examined all the nuclei contained the diploid amount of DNA (e.g., in lymphocytes, kidney, gastric mucosa, and adrenal cortex and medulla) whereas in liver and pancreas

the nuclei fell into three classes with respect to their content of DNA. They differed, however, from Swift in finding that the Class I nuclei of liver contained only 65%, Class II, 115%, and Class III, 323% of the normal diploid amount of DNA. The corresponding percentages for pancreas were: for Class I, 74%; for Class II, 151%; and for Class III, 287%. These results were apparently obtained from a single animal and have never been repeated. Subsequent cytophotometric experiments by Leuchtenberger, Vendrely & Vendrely (1951) and by Frazer & Davidson (1953) have all indicated that in rat liver the Class I nuclei contain the same amount of DNA as the nuclei of rat kidney and that Classes II and III contain respectively twice and four times this amount. Nevertheless the results of Pasteels & Lison constituted an additional reason for investigating the DNA content of individual nuclei in rat liver and pancreas by the cytophotometric method.

4) It has already been shown (Table 25) that the average DNA content per nucleus in rat liver is markedly increased during the phase of very active growth following partial hepatectomy. It has also been shown (Table 24) that the average DNA content per nucleus is lower in livers infiltrated by tumour nodules as a result of administration of dimethylaminoazobenzene than in normal liver. It was clearly desirable to find out what changes in the DNA

content of the different classes of nuclei or in their relative proportions produced these effects on the average content for the whole population. This could only be done by the cytophotometric method.

5) Hitherto attention has been chiefly directed to the question of the existence of significant differences in average DNA content per nucleus between different populations of nuclei. It should, however, be possible to estimate cytophotometrically the degree of variation from one nucleus to another within a single, apparently homogeneous, population of nuclei.

It was concluded that the importance of these five questions was such as to justify a cytophotometric investigation.

3.2 Cytophotometric methods for estimating DNA.

There are at present three methods by which the relative DNA contents of individual nuclei in a tissue section (or a preparation of isolated nuclei) may be estimated.

1). The section may be stained by the method of Feulgen and the amounts of purple stain in the individual nuclei, which are presumed to be proportional to their original contents of DNA, may be measured photometrically. This method appears to have been used first by Stowell in

1942 (Stowell, 1942; see also Stowell, 1947). Its potentialities, however, were not generally realised until Pollister & Ris (1947) devised their simplified cytophotometric apparatus. It has subsequently been applied to the tissues of various mammals (Ris & Mirsky, 1949a; Pollister, 1950; Swift, 1950a; Di Stefano, 1948a, b; Reisner & Korson, 1951; Korson, 1951; Diermeier et al., 1951; Di Stefano, Bass, Diermeier & Tepperman, 1952; Alfert, 1951; Alfert & Bern, 1951; Leuchtenberger, 1950; Leuchtenberger, Vendrely & Vendrely, 1951; Leuchtenberger & Schrader, 1951; Ely & Ross, 1948, 1951a, b; Pasteels & Lison, 1950; Marinone, 1951; Naora, 1951, 1952; Sibatani, Fukuda, Matsuda & Naora, 1952), of the frog (Moore, 1952), of the snail (Leuchtenberger & Schrader, 1952), of the grasshopper larva (Harrington & Koza, 1951), of various mantids (Hughes-Schrader, 1951), of the pentatomid insect Arvelius albopunctatus (Schrader & Leuchtenberger, 1950), of the sea-urchin (Pasteels & Lison, 1951) and of various species of fish (Ris & Mirsky, 1949). It has also been used by Bryan (1951) in a study of microsporogenesis in Tradescantia, by Schrader & Leuchtenberger (1949) and Swift (1950b) in more general investigations of the nuclei of the same species, and by Seshachar (1950) in studies on the ciliate Chilodonella.

A similar method in which methyl green staining is used instead of Feulgen staining has been devised by Kurnick and others (Pollister & Leuchtenberger, 1949; Kurnick, 1950a, b; Kurnick & Mirsky, 1950; see also Devreux, Johansson & Errera, 1951). It has not so far been very widely employed except to confirm or supplement results obtained by the Feulgen method (Di Stefano, 1948a, b; Harrington & Koza, 1951; Korson, 1951; Di Stefano et al., 1952; Frazer & Davidson, 1953) although Kurnick himself has used it in a study of polyteny in the salivary glands of Drosophila (Kurnick & Herskowitz, 1952). Its specificity for DNA appears to depend on the degree of polymerization of the latter substance (Pollister & Leuchtenberger, 1949; Kurnick, 1950a, b; Kurnick & Mirsky, 1950; Vercauteren, 1950) although other factors may also be involved (Taft, 1951; Alfert, 1952).

The validity of both the preceding methods depends entirely on the assumption that the amount of stain present in a nucleus is strictly proportional to its content of DNA. This can, however, be estimated directly by measuring the ultraviolet absorption of the nucleus at about 260 mμ. after removing RNA by treatment with ribonuclease or in some other way. This method, which was originally suggested by Pollister & Ris (1947), has been used by Leuchtenberger, Leuchtenberger, Vendrely & Vendrely (1952), Leuchtenberger,

Klein & Klein (1952) and Frazer & Davidson (1953). (See also Mellors, Keane & Papanicolaou, 1952.)

Those workers who have compared the results obtained for normal tissues by the three methods have generally found good agreement between them (Korson, 1951; Leuchtenberger, Vendrely & Vendrely, 1951; Leuchtenberger, Leuchtenberger, Vendrely & Vendrely, 1952; Di Stefano et al., 1952; Frazer & Davidson, 1953). For the present series of experiments it was decided to use the Feulgen method as described by Frazer & Davidson (1953) since it avoids the technical difficulties associated with ultraviolet microscopy and since in the hands of these workers it gave more satisfactory results than the methyl green method.

3.3 Cytophotometry and the Feulgen reaction.

The theoretical aspects of cytophotometry in general have been extensively studied by Caspersson (see Caspersson, 1950, for a brief review and Caspersson, 1936, for a more detailed treatment). The validity of the method as applied to the estimation of DNA in individual nuclei was first established by Ris & Mirsky (1949) and the principal errors to which it is subject have since been examined by Swift (1950a), Glick, Engstrom & Malmstrom (1951), Moses (1952), Ornstein (1952), Pollister (1952a), Davies &

Walker (1953) and others. These will not be dealt with in detail here except insofar as they have a direct bearing on the results obtained in the present investigation. Since, however, the validity of these depends entirely on the assumption that the amount of purple dye in a Feulgen-stained nucleus is proportional to the amount of DNA which it originally contained, it is necessary, before proceeding farther, to consider how far this assumption may be justified.

The Feulgen technique as applied to histological sections consists essentially of two operations (Feulgen & Rossenbeck, 1924; Baker, 1942; Glick, 1949; Pearse, 1953), viz.,

1) hydrolysis for a short time (usually 5 - 20 minutes) in N hydrochloric acid at 60°C, followed, after washing, by

2) staining with fuchsin-sulphurous acid.

It is generally agreed that the purple colour produced in nuclei and chromosomes by this technique is due to a reaction between the fuchsin-sulphurous acid and the hydrolysis products of DNA. On the basis of adsorption experiments in vitro Carr (1945) put forward the alternative suggestion that it might be due to the chromosomes adsorbing fuchsin molecules so strongly as to displace the sulphurous acid to which the latter were bound and thus restoring their

purple colour. This theory, which implies that DNA is not required for a positive reaction, is not in accordance with the well-known fact that tissue sections will not give a positive Feulgen reaction after treatment with deoxyribonuclease (Brachet, 1946), and, in particular, that this enzyme renders chromosomes Feulgen-negative without destroying their protein structure (Mazia & Jaeger, 1939). It may therefore be rejected. (See also Dobson, 1946; Stowell, 1946.)

The orthodox theory of the mechanism of the Feulgen reaction is that the preliminary hydrolysis splits off purine bases from the DNA molecule thus exposing the aldehyde groups of the corresponding deoxypentose residues (see Osborne & Heyl, 1908). It is presumed that in the second stage of the procedure the fuchsin molecules attach themselves to these aldehyde groups to give a coloured, insoluble addition product (Wieland & Scheuing, 1921; Baker, 1942; Pearse, 1953). On theoretical grounds this hypothesis seems quite reasonable since Tamm, Hodes & Chargaff (1952) have shown that <u>in vitro</u> mild acid hydrolysis does result in a quantitative removal of purine residues from the DNA molecule without completely destroying its highly polymerized structure and that the resultant "apurinic acid" does give an immediate purple colour with fuchsin-sulphurous acid. It is moreover supported by the results of a cyto-

photometric study carried out by Di Stefano (1948a, b) on the Feulgen staining of cartilage nuclei. This author found that as the duration of the preliminary hydrolysis was increased up to a maximum of 12 minutes the intensity of the staining finally produced was also increased. This increase was accompanied by a 50% reduction in the ultraviolet absorption of the nuclei at 260 mμ. due presumably to purine residues being split off from the DNA molecule. Since, however, hydrolysis for less than 12 minutes did not affect the degree to which the nuclei stained with methyl green it was presumed that this "depurination" was not accompanied by depolymerization of the rest of the DNA molecule. If the duration of the hydrolysis was extended from 12 minutes to 24 minutes the ultraviolet absorption of the nuclei at 260 mμ. and their stainability with both fuchsin-sulphurous acid and methyl green decreased almost to zero, indicating that the DNA was being broken down to soluble fragments which were diffusing out of the nuclei. Later experiments by Thomas (1950) have also indicated that during the first 10 minutes of hydrolysis purine residues are removed from the DNA molecule and that the molecule itself is depolymerized only if this hydrolysis time is extended.

More recently, it has become apparent that this is

an oversimplified view of the situation. Stedman & Stedman (1950) have reported that if isolated nuclei are fixed in acetic-alcohol and treated with N hydrochloric acid at 60°C for 10 minutes as in the Feulgen procedure the hydrolysis fluid is subsequently found to give a purple colour with fuchsin-sulphurous acid and a blue colour with the diphenylamine reagent of Dische (1930), indicating that even with this short time of hydrolysis DNA fragments other than free purines are being lost from the nuclei. The magnitude of this loss, which could not be estimated from the data published by Stedman & Stedman (1950), has been systematically investigated by Sibatani & Fukuda (1953). These authors find that it varies quite widely from one experiment to another but when the time of hydrolysis is 13 or 15 minutes it is generally equivalent to about 10% of the deoxypentose of the DNA. It can, as Ely & Ross (1949) have shown, be reduced by cutting down the hydrolysis time, and Brachet (1946) has claimed that if fowl erythrocyte nuclei are hydrolysed for only 5 minutes the hydrolysis fluid does not subsequently give any colour at all with the diphenylamine reagent (i.e., there is no loss of deoxypentose from the DNA of the nuclei).

A totally different theory of the mechanism of the Feulgen reaction has been put forward by Stedman & Stedman

(1943a, b, 1944, 1947a, b, 1950). These workers have found that if a specimen of purified DNA is hydrolyzed with N hydrochloric acid as in the Feulgen procedure and mixed in a test-tube with fuchsin-sulphurous acid a clear purple solution is obtained. If a quantity of chromosomin (the non-histone protein obtained by Stedman & Stedman from isolated nuclei, see Section 1.5 above) is added it adsorbs the dye very strongly and is not decolourised even by repeated washing with water. Stedman & Stedman therefore maintain that when a histological preparation is stained by the Feulgen method the preliminary hydrolysis converts the DNA to soluble diffusible fragments. These, in turn, combine with the fuchsin-sulphurous acid to give a soluble diffusible dye which is adsorbed by the chromosomin of the nucleus. On this view, therefore, the structures which stain most intensely by the Felugen method are not those which contain DNA but those which contain chromosomin.

This seems a rather implausible theory. There can be little doubt that the preliminary hydrolysis does degrade some of the DNA to soluble diffusible fragments capable of giving a purple colour with fuchsin-sulphurous acid but most of these fragments must diffuse out into the hydrolysis fluid, where they have been demonstrated by Ely & Ross (1949), by Sibatani & Fukuda (1953) and by Stedman &

Stedman (1950) themselves. The remainder must almost certainly be washed out when the preparation is rinsed after hydrolysis and before it is treated with fuchsin-sulphurous acid. Moreover, if the Stedman theory is correct it is extremely difficult to see why the Feulgen method should stain precisely those structures in the cell which can be shown by other methods (e.g., ultraviolet absorption measurements) to contain nucleic acid (Callan, 1943; Caspersson, 1944; Barber & Callan, 1944). The only real evidence which can be produced in support of the Stedman theory is that the purple dye produced by mixing fuchsin-sulphurous acid in vitro with hydrolyzed DNA ("developed nucleal stain") can be used histologically to stain chromosomes (Choudhuri, 1943; Danielli, 1947). The results obtained by this technique are, however, quite different from those obtained by the Feulgen method proper since the cytoplasm is also stained (Danielli, 1947). In an attempt to test the Stedman theory experimentally Brachet (1946) fixed purified specimens of DNA with alcohol or Zenker and embedded them in agar-paraffin. Sections of these preparations were cut in the usual manner and stained by the Feulgen method. On microscopie examination it was found that the particles of DNA had stained intensely and that in the preparations fixed in Zenker the coloration was perfectly

localized. Alcohol-fixed preparations showed slight diffusion. In later experiments on somewhat similar lines Lessler (1951) confirmed Brachet's finding that the Feulgen method stains DNA *in situ*. It seems, therefore, that the balance of the available evidence is strongly against the mechanism postulated by Stedman & Stedman.

A fourth theory of the mechanism of the Feulgen reaction has been put forward by Stacey and his associates (Li & Stacey, 1949; Overend & Stacey, 1949; Overend, 1950; Lee & Peacocke, 1952). This group had originally suggested that ω-laevulaldehyde might be the hydrolysis product of DNA which combined with fuchsin-sulphurous acid to give the coloured complex (Deriaz, Stacey, Teece & Wiggins, 1946). Subsequently, realising that an aldehyde of such low molecular weight would be washed out of a histological preparation before the stage of staining with fuchsin-sulphurous acid was reached and could not therefore be responsible for the observed reaction, they have re-investigated the question. The results of their chemical studies carried out on purified specimens of sperm DNA suggest that the aldehyde groups with which the fuchsin-sulphurous acid molecules combine may be unmasked by two distinct reactions,

1) a "depurination" of the type postulated by the orthodox theory, and
2) a hydrolysis of polymeric deoxypentose-1-phosphate linkages which Stacey believes to be concerned in maintaining DNA in its maximum macromolecular state.

Evidence has been produced which indicates that in the Feulgen method as it is usually performed both reactions would result in DNA being stained in situ. The second reaction, which occurs rapidly under very mild conditions is presumably of relatively greater importance when short hydrolysis times are used.

It would be unwise to assume that even Stacey's theory can offer a complete description of the mechanism of the Feulgen reaction. It provides, for example, no explanation of the fact that when a purified specimen of DNA is hydrolyzed and stained with fuchsin-sulphurous acid in vitro the intensity of the colour developed is modified to some extent by the presence of histones or other proteins (Widstrom, 1928; Caspersson, 1932; Sibatani, 1950; Moses, 1951; Sibatani, Fukuda, Matsuda & Naora, 1952). Clearly, further investigation will have to be undertaken to elucidate this phenomenon.

Nevertheless, in spite of the apparent complexity of the reaction, it has been shown by Ris & Mirsky (1949a)

that if sections of tissue from different species are mounted on the same slide and stained together by the Feulgen method, then the average amount of purple stain per nucleus, estimated cytophotometrically, for the different tissues is almost exactly proportional to the average DNA content per nucleus, as determined by chemical analysis of counted suspensions of isolated nuclei. Similar results have since been reported by other workers (Leuchtenberger, Vendrely & Vendrely, 1951; Pollister, 1952b; Pollister, Swift & Alfert, 1951). It seems therefore justifiable, on purely empirical grounds, to assume that the relative amounts of Feulgen stain in different nuclei on the same slide are proportional to the amounts of DNA which these nuclei originally contained.

3.4 Experimental methods.

Animals. The animals used were male albino rats weighing 200 - 300 g. from the departmental colony. They were maintained on a stock diet of "rat-cake" (diet (a) in Section 2.2) with the exception of one animal which received the carcinogenic diet previously described (diet (g) in Section 2.2) for 14 weeks before being sacrificed. A second animal was subjected to partial hepatectomy (removal of median and left lateral lobes) by the method of Higgins & Anderson (1931). The animals were killed by exsanguina-

tion under ether anaesthesia and the tissues required rapidly removed and, if necessary, stored in solid carbon dioxide.

Isolation of nuclei. Since the Feulgen reaction stains only the nuclei leaving the cytoplasm clear and colourless the DNA content of individual Feulgen-stained nuclei may be measured cytophotometrically either in situ in a tissue section or in a smear preparation of isolated nuclei. The use of tissue sections has the obvious advantage that it makes possible the classification of each nucleus measured according to the cell type to which it belongs. On the other hand, since a tissue section can seldom have a thickness much greater than the average diameter of the nuclei which it contains there is a danger that measurements may be made on nuclei which have been "chipped" by the microtome blade during sectioning. In order to avoid this difficulty it was decided in the present investigation to carry out measurements only on smear preparations of isolated nuclei. The method of isolation used was as follows:

The tissue, if fresh, was chilled in ice or, if frozen, was allowed to thaw in the refrigerator. It was then finely minced with scissors and homogenized for about two minutes in an ice-jacketted Nelco blendor in a suitable

volume (usually about 40 ml.) of ice-cold 0.25M sucrose containing 0.008M citric acid. The homogenate was strained once or twice through a double layer of fine nylon gauze and centrifuged at 0°C for 10 minutes at 880 g. The supernatant was discarded and the sediment resuspended in 30 - 40 ml. ice-cold sucrose/citric acid solution and centrifuged at 500 g. for 5 - 7 minutes. The supernatant was discarded and the process of resuspending the sediment and centrifuging down again was repeated once or twice to get rid of cytoplasmic débris. Finally, the sediment was freed from sucrose by resuspending in 0.01M citric acid and centrifuging down. The product obtained by this technique generally consisted of free nuclei more or less heavily contaminated with unbroken whole cells and cytoplasmic granules. In the case of pancreas, only a few free nuclei were obtained together with a large number of whole cells. Since under the conditions of the Feulgen reaction this cytoplasmic material did not stain, its presence was of no importance. The isolation procedure was not, as in the previous series of experiments, intended to yield a suspension of isolated nuclei free, so far as possible, from all cytoplasmic contamination. On the contrary, its real object was twofold:

(1) to obtain large numbers of intact nuclei which could be used to make a smear preparation suitable for cytophotometry; and

(2) to ensure that when this preparation was fixed and stained the purple stain should be evenly distributed within each nucleus. This was achieved by the use of sucrose in the isolation medium (Ris & Mirsky, 1949a).

It should be noted that Ris & Mirsky (1949b) have shown that no DNA is lost from nuclei isolated in sucrose solution even if they remain overnight in this medium (see also Arnesen, Goldsmith & Dulaney, 1949).

Preparation of slides. Smear preparations of the "isolated nuclei" obtained by the procedure described above were made on microscope slides and allowed to dry. A similar preparation of rat spermatozoa was obtained by dividing the vas deferens close to the prostate gland, expressing a drop of seminal fluid from the distal end on to a slide, spreading it with a glass rod, and allowing it to dry. A smear of normal kidney nuclei was made on each slide as a control. The slides were fixed in a 3 : 1 mixture of ethanol and acetic acid for 30 minutes at room temperature, washed in running tap water for 2 hours, and allowed to dry.

Feulgen staining. The following reagents were used:

A N hydrochloric acid.

B Fuchsin-sulphurous acid stain prepared by Coleman's (1938) modification of the method of de Tomasi (1936).

1 g. basic fuchsin (British Drug Houses Ltd.) was dissolved in 200 ml. boiling water with vigorous shaking. When the solution had cooled to 50°C it was filtered and 2 g. potassium metabisulphite and 10 ml. N hydrochloric acid were added. After standing for 24 hours the solution was decolourised by adding 0.5 g. animal charcoal, shaking vigorously for one minute, and filtering rapidly. The resultant colourless, water-clear solution was stored in a tightly stoppered bottle in the refrigerator.

C Acid metabisulphite solution prepared by the method of 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 staining technique used was as follows. The slides were rinsed in cold N hydrochloric acid, placed in N hydrochloric acid at 60°C for 6 minutes, rinsed again in cold N hydrochloric acid and transferred for a few seconds to distilled water. They were then placed in the fuchsin-sulphurous acid stain at room temperature for 2 hours. At the end of this period they were removed, allowed to drain, and transferred to the acid metabisulphite solution for 1 hour. Finally they were rinsed in running water, dried in

air, and mounted in DPX neutral medium.

Photomicrography. Photomicrography was carried out by the method of Frazer & Davidson (1953). A type M 8001 ultraviolet microscope manufactured by Cooke, Troughton & Simms Ltd. was used in conjunction with a 35 mm. camera without lens. The camera was interchangeable with a ground glass screen for focussing. The optical system was as follows: 3.75 mm. fluorite objective, N.A. 0.95, immersion liquid 85% glycerol; 10X ocular, distance from ocular to film 22 cm. The substage condenser was quartz, glycerol immersion, N.A. 1.25, iris aperture 6 mm. Green light of wavelength 546 mμ. was obtained by using a high pressure mercury arc in conjunction with a colour filter (Hilger "mercury green") and a single quartz prism. The nuclei were photographed on Ilford type F.P.3 film which was developed for 10 minutes in Kodak D76 developer at 18°C. A positive print of a typical field of nuclei photographed under these conditions is shown in Fig.4.

A separate strip of film was used for each slide. In general, 10 to 30 fields of the nuclei under examination were photographed together with 6 to 10 fields of the control smear of kidney nuclei on the same slide. A logarithmic step-wedge was photographed on to each strip of film by placing the camera behind a rotating step-sector in the same

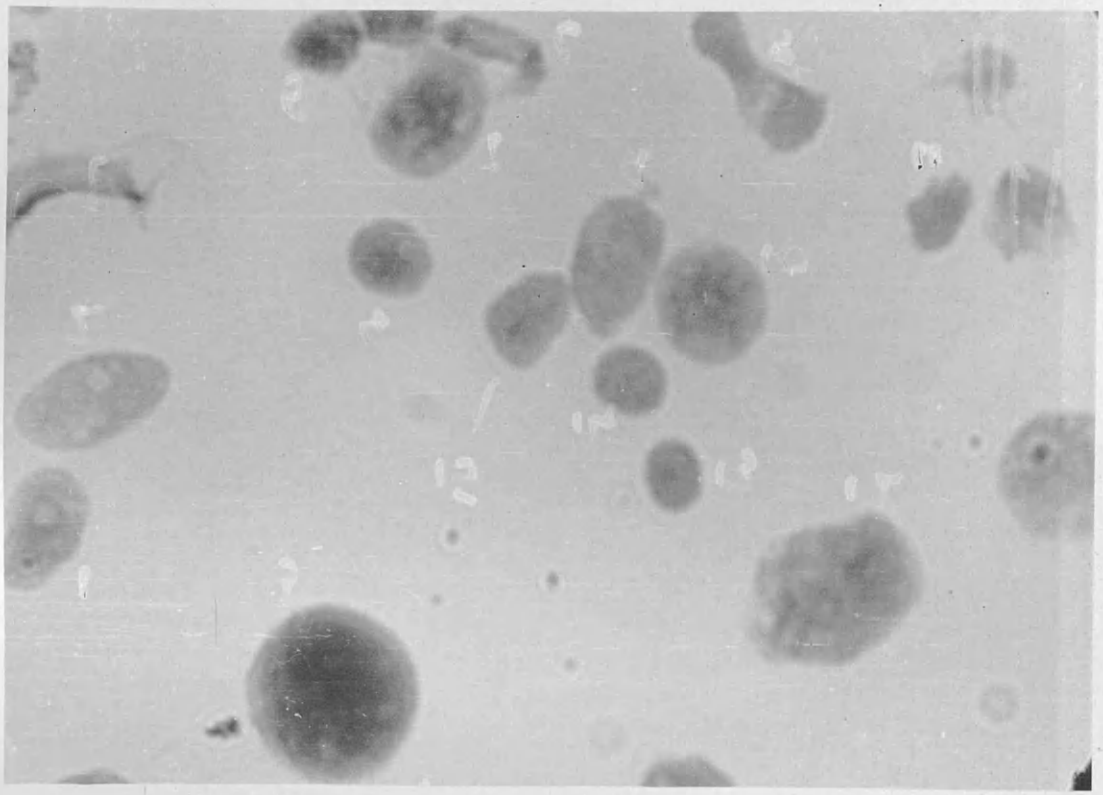

Figure 4.

Figure 5.

Figure 6.

Positive print of a small nucleus, photographed using "two-spot" illumination as described in Section 3.4 (enlarged x 6 from original negative used for cytophotometry). The nucleus appears as a small dark disc which does not quite fill the lower of the two illuminated areas. The other illuminated area (the "background spot") includes only "empty" background (see pp.148-149).

Figure 7.

Positive print of typical field of rat spermatozoa, stained and photographed as described in Section 3.4 (enlarged x 6 from original negative used for cytophotometry). Only the nuclei are actually stained but the tails, which are highly refractible, are also visible.

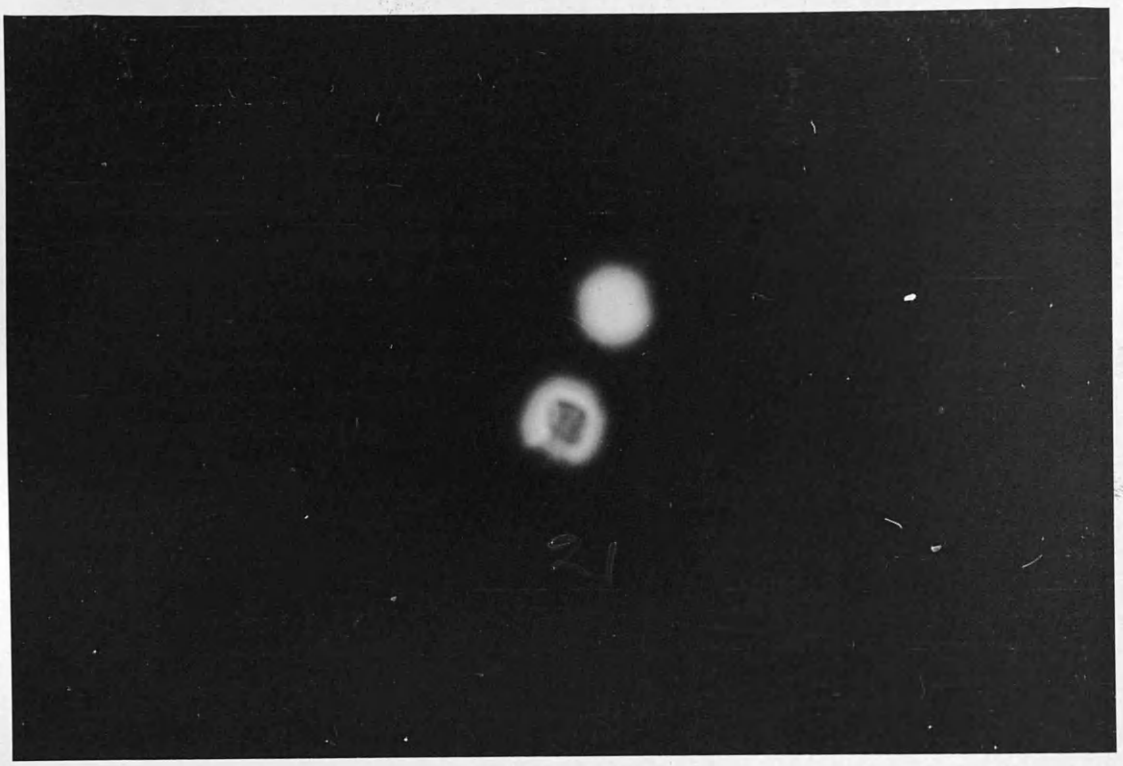

Figure 6.

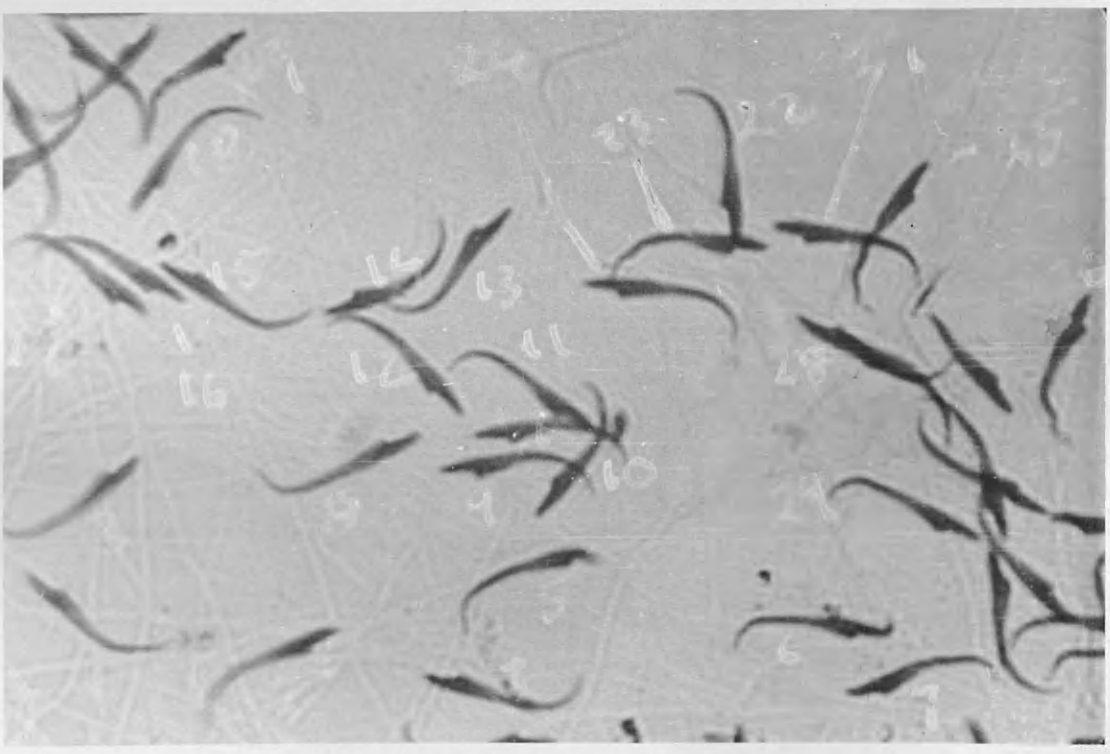

Figure 7.

light path as that used for the microscope. The construction of the step-sector was such that the area of film immediately behind the first step was occluded for $^{63}/_{64}$ of the total time of exposure; the area behind the second step was occluded for $^{31}/_{32}$ of the total time of exposure; the area behind the third step for $^{15}/_{16}$; the area behind the fourth step for $^{7}/_{8}$; the area behind the fifth step for $^{3}/_{4}$; and the area behind the sixth step for $^{1}/_{2}$. These six areas formed the first six steps of the step-wedge. The seventh was formed by an adjacent area of film which was not occluded at all by the rotating step-sector. Fig.5 shows a positive print of such a step-wedge taken from the same film-strip as the field of nuclei shown in Fig.4. Exposure times for the nuclei and step-wedge were chosen by trial and error so as to give a suitable range of optical densities.

Lenticular glare. The arrangement of light source, and substage condenser described above was such as to illuminate evenly the entire field photographed. It has been claimed by Naora (Naora, 1951, 1952; Naora & Sibatani, 1952) that under these conditions the light falling on the image of a nucleus on the film consists not only of light which has been transmitted through that nucleus but also of light which has passed through other areas of the slide and has been scattered by internal reflection from the lens

surfaces within the microscope (lenticular glare). If this is, in fact, the case the percentage of incident light transmitted by the nucleus will be overestimated, i.e., its optical extinction will be underestimated. Naora believes this so-called Schwarzschild-Villiger effect to be a serious source of error in cytophotometry and has pointed out that it may be eliminated by so arranging the light source and substage condenser that only the nucleus being measured (or photographed) is illuminated (Naora, 1951, 1952; Naora & Sibatani, 1952). Accordingly a lighting system of this description was devised in this laboratory by Dr. S.C.Frazer. The effective size of the light source was diminished by placing in front of it a blackened metal plate in which two holes approximately 1 mm. in diameter had been drilled. The image of these perforations was focussed on the microscope slide using, in place of the normal substage condenser, a high power Leitz microscope objective. The exact optical specification of this objective is unfortunately unknown but it was used without immersion liquid and because of the shortness of its working distance it could only be employed with a slide less than 1 mm. thick. In this way it was possible to limit the area of the slide illuminated to two circular areas approximately 10 μ. in diameter, and thus to photograph fields in which only a single nucleus and a

separate but adjacent clear area of the slide were illuminated. (A positive print taken from a film strip in which this method of illumination was used is shown in Fig.6.) In one experiment the results obtained by this method of "two-spot illumination" were compared with those found by the ordinary "whole-field illumination" method used in all other experiments.

Measurements of area and optical extinction. Each nucleus on the negative was numbered, those which were obviously damaged, or which overlapped, or which were obviously out of focus being rejected. The negatives were projected to 30 - 40 diameters enlargement in a film strip projector, the outline of each nucleus was drawn, and the area of the drawing subsequently measured in arbitrary units with a planimeter. This figure was taken as the area (A) of the nucleus. Densitometer measurements were also made on the negative, using a Hilger spectrograph-plate densitometer. Readings were taken for each step of the step-wedge, for three small areas within the image of each nucleus, and for two equal areas of "empty" background adjacent to each nucleus (or, where "two-spot illumination" had been used, on two equal areas of the background spot, see Fig.6).

Calculation of the amount of absorbing material in each nucleus. The densitometer readings obtained from the step-wedge were used to construct a calibration curve for

the film strip of densitometer readings against film exposure. This was done by assuming that the first step of the step-wedge, which had been occluded for $^{63}/_{64}$ of the total time of exposure (see above), had been exposed to 1 arbitrary unit of light. It followed that the second step, which had been occluded for $^{31}/_{32}$ (= $^{62}/_{64}$) of the total time of exposure, had been exposed to 2 arbitrary units; the third step, which had been occluded for $^{15}/_{16}$ (= $^{60}/_{64}$) of the total of exposure, had been exposed to 4 arbitrary units; the fourth step, which had been occluded for $^{7}/_{8}$ (= $^{56}/_{64}$) of the total time of exposure, had been exposed to 8 arbitrary units; the fifth step, which had been occluded for $^{3}/_{4}$ (= $^{48}/_{64}$) of the total time of exposure, had been exposed to 16 arbitrary units; the sixth step, which had been occluded for $^{1}/_{2}$ (= $^{32}/_{64}$) the total time of exposure, had been exposed to 32 arbitrary units; and the seventh step, which had not been occluded by the rotating step-sector at all, had been exposed to 64 arbitrary units. Using this calibration curve it was possible to convert the mean of the three densitometer measurements taken inside the nucleus into an estimate of T the intensity of the light transmitted by the nucleus; and the mean of the two measurements taken in areas of "empty" background adjacent to the nucleus into an estimate of I the intensity of light

reaching the area of film surrounding the nuclear image. Then the extinction (E) of the nucleus is given by

$$E = \log_{10} {}^{I}/_{T}$$

and in spherical or ellipsoidal nuclei the amount of absorbing material present may be taken as proportional to the product E A (Frazer & Davidson, 1953). It should be noted that the units in which E A is expressed are quite arbitrary and vary in magnitude from one experiment to another. Hence comparisons of numerical values can only be made internally in one experiment.

An example, taken from an actual experiment, may make the method of calculation clearer. Figs.5 and 4 are positive prints of, respectively, the step-wedge and a typical field of nuclei photographed on the same strip of film during the same experiment. The densitometer readings obtained for the step-wedge were as follows:

For the 1st step, exposed to 1 arbitrary unit of light, 165

" " 2nd " " " 2 " units " " , 146

" " 3rd " " " 4 " " " " " , 123

" " 4th " " " 8 " " " " " , 94

" " 5th " " " 16 " " " " " , 61

" " 6th " " " 32 " " " " " , 38

" " 7th " " " 64 " " " " " , 22.

Figure 8.

Calibration curve of densitometer reading against arbitrary units of film exposure (see pp.149 - 151).

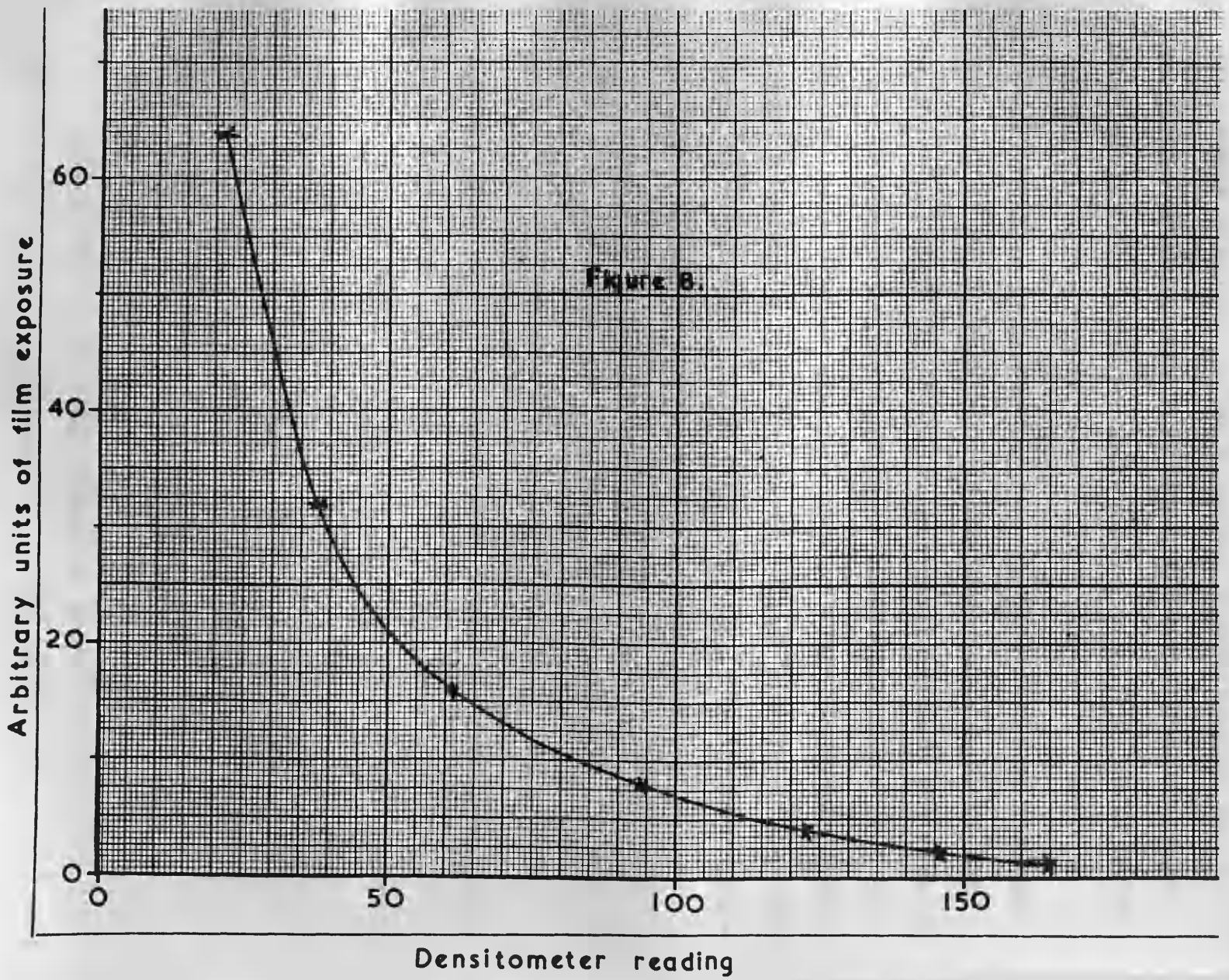
Arbitrary units of film exposure
60
40
20
0
0
50
100
150
Densitometer reading

Figure 8.

From these readings the calibration curve shown in Fig.8 was drawn relating the densitometer reading for any given area of the developed negative to the amount of light which had fallen on that area.

The three densitometer readings obtained for nucleus number 3 in Fig.4 were 96, 105 and 93. Referring to the calibration curve in Fig.8, it will be seen that the mean of these readings, 98, is equivalent to a value for T of 7.4. The two densitometer readings obtained for the "empty" background adjacent to nucleus number were 31 and 33. Referring again to Fig.8, it will be seen that the mean of these readings, 32, is equivalent to a value for I of 41.5.

Then for this nucleus $E = \log_{10} {}^{I}/_{T}$

$= \log_{10} {}^{41.5}/_{7.4}$

$= 0.749.$

But the area (A) of nucleus number 3 was found by planimetry to be 6830 arbitrary units.

Therefore DNA content of nucleus number 3 was 0.749 x 6830 = 5120 arbitrary units.

Similarly, for nucleus number 2 in Fig.4.

Densitometer readings taken within nucleus were 40, 49 and 39, mean = 42.7. Therefore, from Fig.8, T = 26.5 arbitrary units.

Densitometer readings taken in areas of empty background adjacent to the nucleus were 32 and 32, mean = 32. Therefore,

from Fig.8, I = 41.5 arbitrary units.

Then, for this nucleus, $E = \log_{10} {}^{I}/_{T}$

$= \log_{10} {}^{41.5}/_{26.5}$

$= 0.195.$

But the area (A) of nucleus number 2 was found by planimetry to be 2940 arbitrary units. Therefore DNA content of nucleus number 2 was 0.195 x 2940 = 574 arbitrary units.

It must again be emphasized that these figures indicate only that the DNA contents of nuclei numbers 2 and 3 are in the approximate ratio 574 : 5120. They give no indication of the absolute DNA content of the nuclei. Nor can they be directly compared with the figures obtained in other experiments of the same type.

Chemical estimation of average DNAP content per nucleus.

In certain cases it was thought desirable, in addition to estimating the relative DNA content of individual nuclei in a tissue, to determine its average DNAP content per nucleus in absolute units by the technique of gross chemical analysis of counted suspensions of isolated nuclei described in Section 2.2. This was done by mincing the tissue finely with scissors and dividing the mince into two portions, one of which was used for cytophotometry while from the other nuclei were isolated for chemical analysis. Since it had been found in the previous investigation (Section 2.3) that the three chemical methods used to estimate DNA

(phosphorus determination, deoxypentose determination, and ultraviolet absorption measurements) gave very similar results, only the phosphorus determination method was used in the present series of experiments. Similarly, in the case of other tissues, where the average DNAP content per nucleus previously determined by the chemical method (Table 20) is cited, only the figure obtained by the phosphorus determination method is given.

3.5 Results.

Normal liver. The results obtained in an experiment in which the DNA contents of 116 kidney and 479 liver nuclei were measured are shown, in the form of frequency histograms, in Fig.9. The kidney nuclei form a single symmetrical peak extending from 280 to 720 units. Only two nuclei, which have DNA contents of 920 - 1080 units, fall outside this group. The mean DNA content for all the kidney nuclei measured is 509 units. The values found for liver nuclei, on the other hand, range from 280 to 2520 units of DNA with a mean of 783 units. By this method, therefore, the ratio

$$\frac{\text{Mean DNA content of liver nuclei}}{\text{" " " " kidney "}} = \frac{783}{509} = 1.54$$

In the previous investigation it was found by chemical analysis of counted suspensions of isolated nuclei that the average DNAP content per nucleus was about 0.91 pg. in

Figure 9.

Frequency histograms of the relative deoxyribonucleic acid (DNA) contents of individual rat kidney and liver nuclei as estimated by the cytophotometric method described in Section 3.4.

Class interval = 40 arbitrary units.

N.B. The arbitrary units used in this Figure are not identical with those used in Figs.10, 11, 12, 13, 14 or 15, or in Tables 64 or 66.

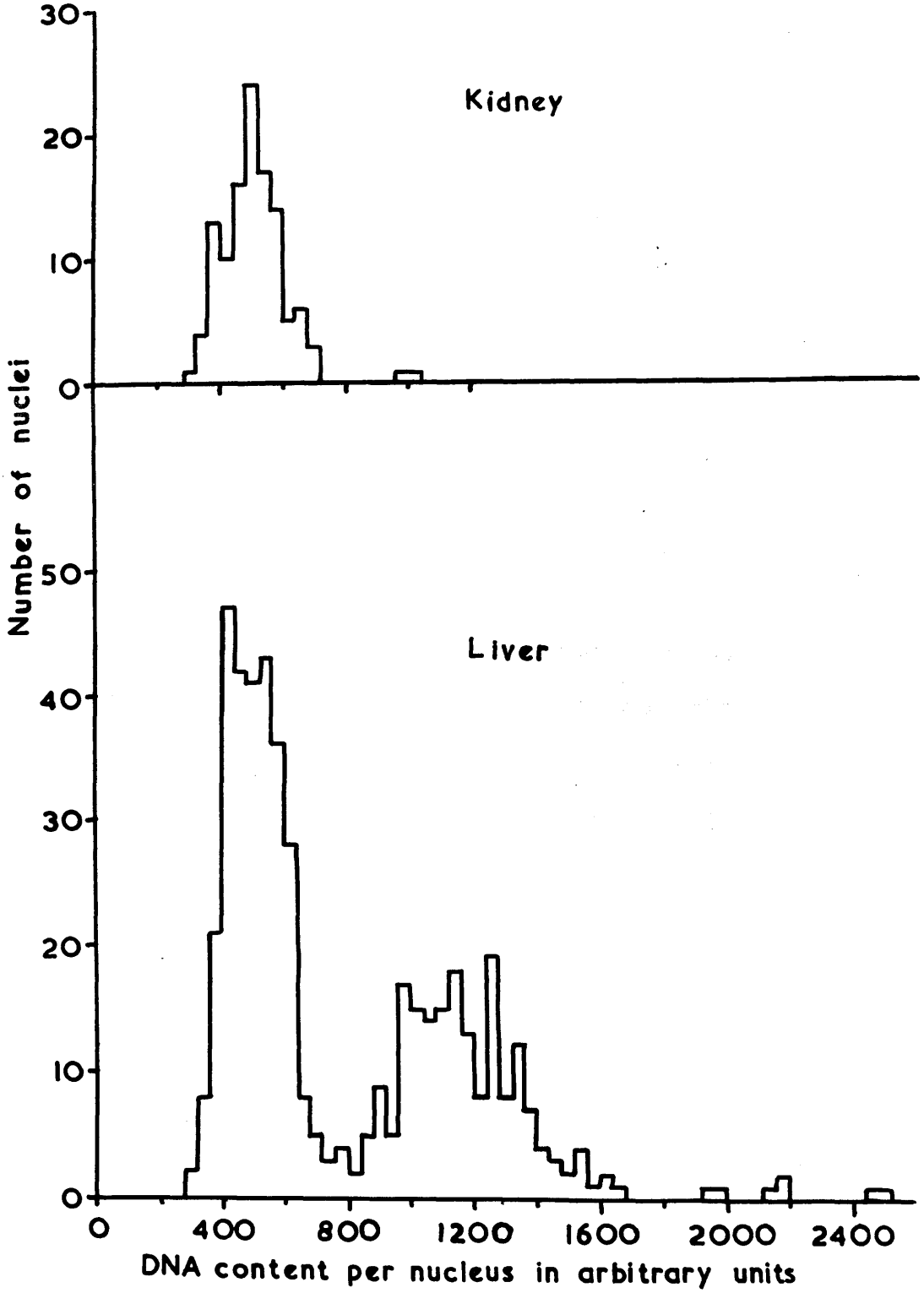
Kidney
Liver
Number of nuclei
30
20
10
0
50
40
30
20
10
0
0
400
800
1200
1600
2000
2400
DNA content per nucleus in arbitrary units

Figure 9.

liver and 0.65 pg. in kidney. By the chemical method, therefore,

$$\frac{\text{Mean DNA content of liver nuclei}}{\text{" " " " kidney "}} = \frac{0.91}{0.65} = 1.40$$

The moderately good agreement between the ratios obtained by the two methods appears to justify the assumption that the cytophotometric method used in conjunction with Feulgen staining is a fairly reliable means of estimating the relative DNA contents of nuclei.

It is apparent from Fig.9 that the liver nuclei fall into two or, more probably, three groups. The largest group, which in accordance with convention (Swift, 1950a; Pollister, Swift & Alfert, 1951) we may term Class I, extends from 280 to about 700 - 800 units and clearly corresponds to the single kidney peak. The second main group, Class II in Swift's (1950a) terminology and consisting presumably of tetraploid nuclei, covers the range from about 700 - 800 units to 1680 units. It may be presumed that the two kidney nuclei with DNA contents of about 1000 units also belong to this Class. Finally, the 7 liver nuclei which gave values of 1820 to 2520 units of DNA presumably represent Swift's (1950a) Class III or octoploid nuclei. If the upper limit of the Class I kidney nuclei, 720 units, can be taken as the boundary between the Class I

and Class II nuclei in the liver the 479 liver nuclei measured may be divided into

281 Class I nuclei, i.e., 58.7% of the whole sample, with a DNA content of 280 to 720 units, mean = 502 units;

191 Class II nuclei, i.e., 39.9% of the whole sample, with a DNA content of 720 to 1680 units, mean = 1145 units; and

7 Class III nuclei, i.e., 1.46% of the whole sample, with a DNA content of 1920 to 2520 units mean = 2197 units.

The mean DNA content of the Class I kidney nuclei is 500 units, i.e., virtually the same as for the Class I liver nuclei. It will also be observed that the mean DNA contents of the three Classes of nuclei in the liver are approximately in the ratio 1 : 2 : 4. These observations are in agreement with the findings of Ris & Mirsky (1949), Leuchtenberger, Vendrely & Vendrely (1951), Leuchtenberger, Leuchtenberger, Vendrely & Vendrely (1952), Frazer & Davidson (1953) and Swift (1950a) previously discussed (see Sections 2.4 and 3.1). The relative proportions of the different classes of nuclei in the liver as estimated in the present experiment may be compared with the relative proportions of diploid and polyploid nuclei as determined histologically by other workers (see Table 63). The moderately good agreement be-

Table 63.

The relative proportions of diploid, tetraploid, octoploid and 16-ploid nuclei in normal rat liver as estimated histologically by various workers (see also Section 2.4).

Reference	% diploid	% tetraploid	% octoploid	% 16-ploid
Biesele (1944)	54.5	40.5	5.0	-
*Sulkin (1943)	47.4	47.5	4.9	0.2
*McKellar (1944)	58.0	42.0	-	-

*Recalculated on the assumption that the hepatocytes account for only 60% of the nuclei in the liver and that the non-hepatocyte nuclei are all diploid (see Section 2.4).

tween the histological and cytophotometric results would appear to indicate that the nuclei measured in the present experiment constituted a fairly representative sample of the whole population of nuclei in the liver.

Lenticular glare. Reference has already been made (Section 3.4) to the claim of Naora (Naora, 1951, 1952; Naora & Sibatani, 1952) that lenticular glare may introduce large errors into the results obtained by cytophotometry. Fig.10 shows the results obtained by measuring the DNA content of 72 liver nuclei (a) using normal "whole-field" illumination and (b) using "two-spot" illumination which should virtually eliminate lenticular glare. It is clear that, irrespective of the type of illumination used, the results fall into essentially the same bimodal distribution as that shown in Fig.9. (It must, of course, be borne in mind that, as has already been pointed out above, the arbitrary units shown in Fig.9 are not necessarily of the same magnitude as those shown in Fig.10.) It may therefore be presumed that the use of "whole-field" illumination throughout the present series of experiments has not resulted in the introduction of any very serious errors.

Pancreas and intestine. Fig.11 shows the results obtained in an experiment in which the DNA contents of nuclei isolated from kidney, pancreas and small intestine were

Figure 10.

Frequency histograms of the relative deoxyribonucleic acid (DNA) contents of 72 rat liver nuclei measured (a) using "whole-field" illumination and (b) using "two-spot" illumination.

Class interval = 100 arbitrary units.

N.B. The arbitrary units used in this Figure are not identical with those shown in Figs. 9, 11, 12, 13, 14 or 15, or in Tables 64 or 66.

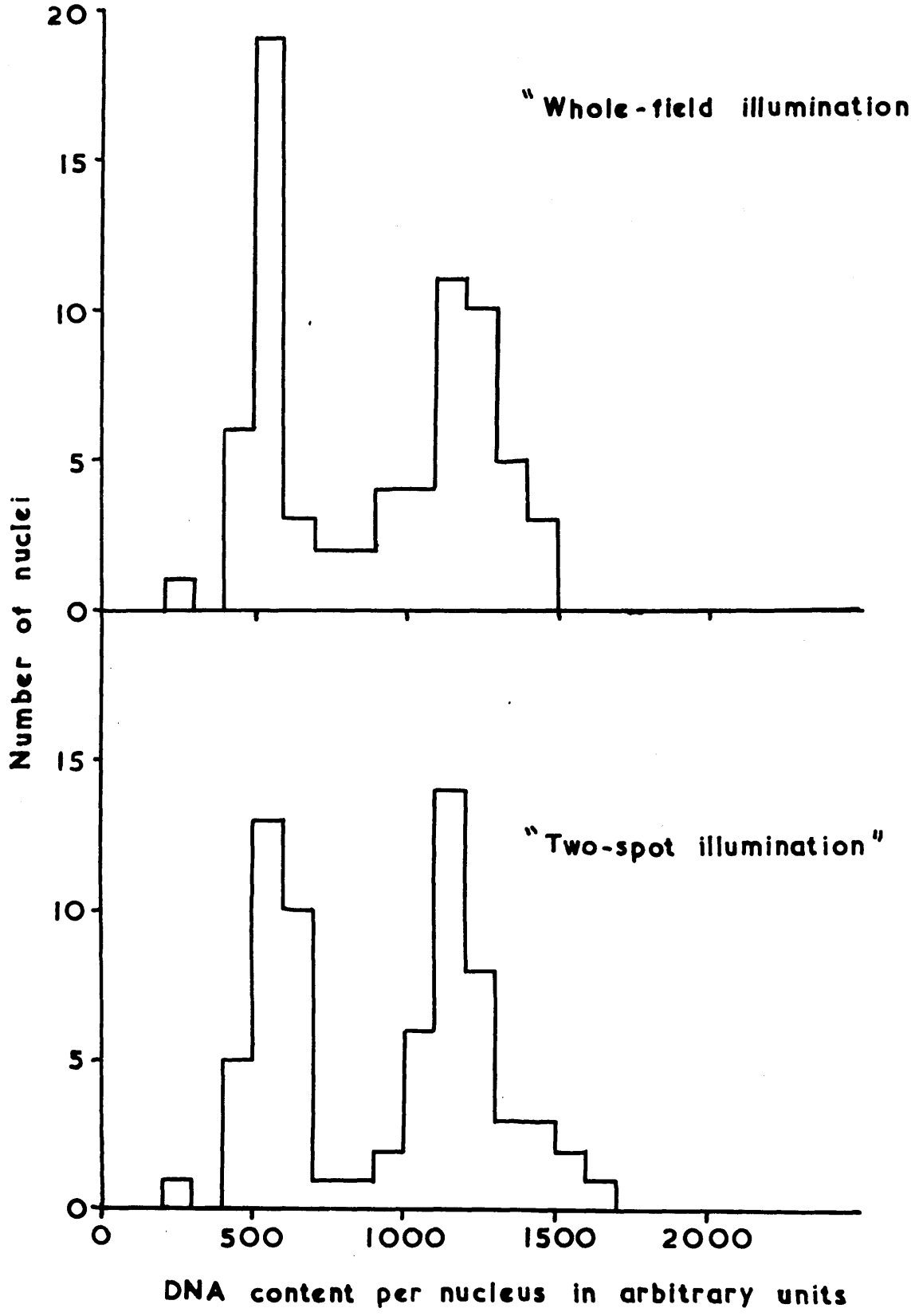
"Whole-field illumination"
"Two-spot illumination"
Number of nuclei
20
15
10
5
0
15
10
5
0
0
500
1000
1500
2000
DNA content per nucleus in arbitrary units

Figure 10.

Figure 11.

Frequency histograms of the deoxyribonucleic acid (DNA) contents of individual rat kidney, small intestine, and pancreas nuclei as estimated by the cytophotometric method described in Section 3.4.

Class interval = 20 units.

N.B. The arbitrary units used in this Figure are not identical with those used in Figs. 9, 10, 12, 13, 14 or 15, or in Tables 64 or 66.

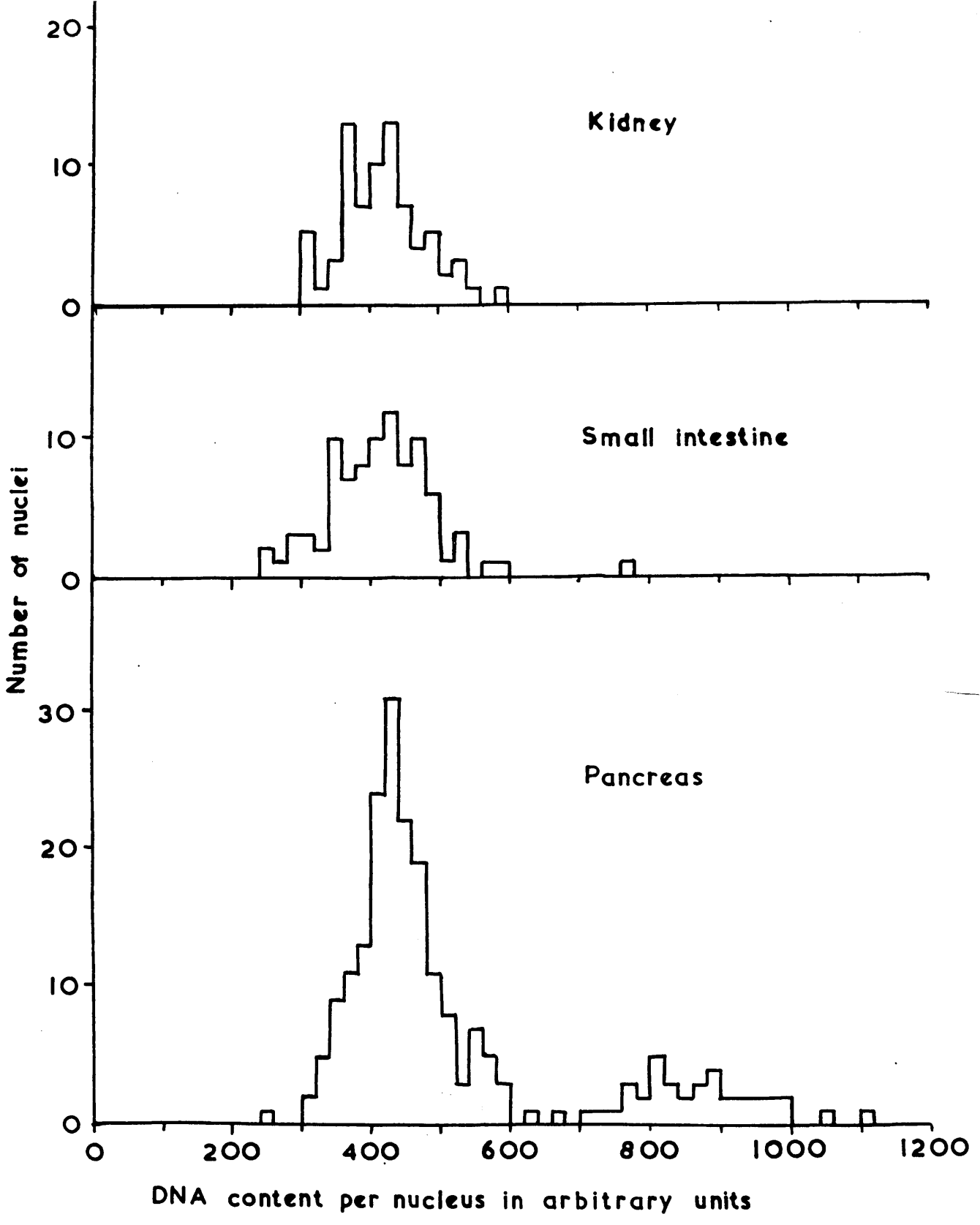
Kidney
Small intestine
Pancreas
Number of nuclei
DNA content per nucleus in arbitrary units
0
10
20
30
200
400
600
800
1000
1200

Figure 11.

measured. The mean DNA content of all the kidney nuclei measured is 419 units; of all the small intestine nuclei, 411 units; and of all the pancreas nuclei, 515 units. The means for the three tissues therefore fall in the ratio 1 : 0.98 : 1.23. The approximate average DNAP contents per nucleus found by chemical analysis in the previous investigation (Table 20) were: for kidney, 0.65 pg.; for small intestine, 0.74 pg.; and for pancreas, 0.71 pg. These chemically determined figures are in the ratio 1 : 1.14 : 1.09. As in the case of liver and kidney, the moderately good agreement of these ratios determined by two totally different methods supports the assumption that the cytophotometric technique used in the present series of experiments is a fairly reliable means of estimating the relative DNA content of nuclei.

It is clear from Fig.11 that all the 75 kidney nuclei and 87 of the 88 small intestine nuclei belong to Class I. The remaining intestine nucleus probably belongs to Class II. Pancreas, on the other hand, appears to contain a considerable proportion of Class II nuclei. If the figure of 600 units, which marks the upper limit of the Class I nuclei of kidney and intestine, is taken as the boundary between Class I and Class II nuclei in pancreas, the 213 pancreas nuclei measured may be divided into

174 Class I nuclei, i.e., 81.7% of the whole sample, with a DNA content of 240 to 600 units, mean = 439 units;

and 39 Class II nuclei, i.e., 18.3% of the whole sample with a DNA content of 620 to 1120 units, mean = 858 units.

(It should again be noted that the arbitrary units used in Fig.11 are not identical with those used in Figs.9 or 10.) As in the case of liver the mean DNA contents of Classes I and II are in the approximate ratio 1 : 2 and the mean DNA content of Class I pancreas nuclei is very close to that found for Class I kidney nuclei. Similar results have been obtained by Swift (1950a) for mouse pancreas. It is of some interest that Jacobj's (1925) measurements of nuclear volumes suggest that about 30% of the parenchymal cells of rat pancreas are tetraploid.

Spermatozoa. Fig.12 shows the results obtained by comparing the DNA contents of rat sperm heads and rat kidney nuclei. (Again the arbitrary units used in Fig.12 are not the same as those used in Figs.9, 10 or 11.) The kidney nuclei measured all appear to belong to Class I. Their average DNA content is 337 units. The sperm heads also fall into a single group with an average DNA content of 195 units. The ratio, mean DNA content of sperm heads/mean

Figure 12.

Frequency histograms of deoxyribonucleic acid contents of individual rat spermatoxoa and kidney nuclei as estimated by the cyclophotometric method described in Section 3.4.

Class interval = 20 arbitrary units.

N.B. The arbitrary units used in this Figure are not identical with those used in Figures 9, 10, 11, 13, 14 or 15, or in Tables 64 or 66.

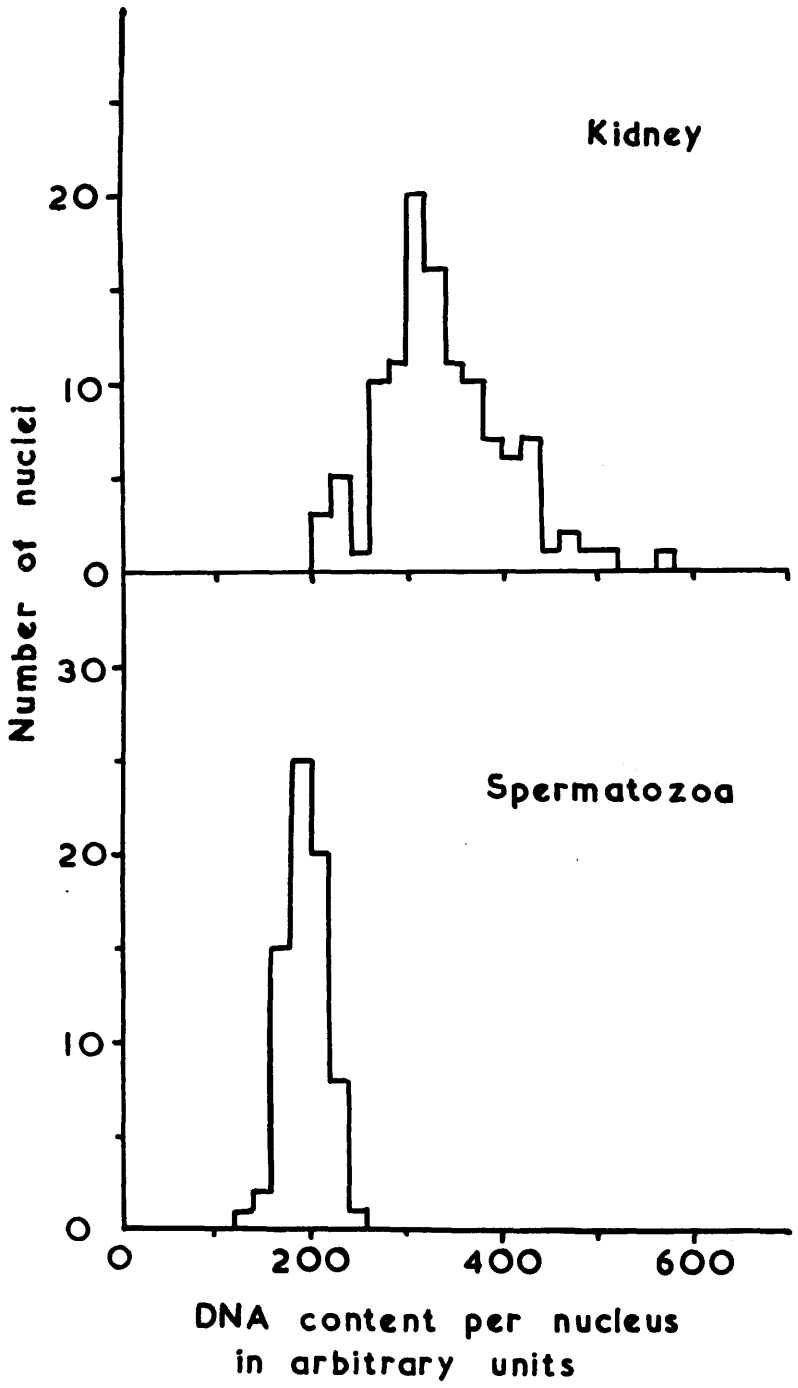
Kidney
20
10
0
Number of nuclei
30
Spermatozoa
20
10
0
0
200
400
600
DNA content per nucleus
in arbitrary units

Figure 12.

DNA content of kidney nuclei is thus 195/337 = 0.59 : 1.00 instead of 0.50 : 1.00 as predicted by the Boivin-Vendrely hypothesis. This discrepancy can, however, probably be fully accounted for by the errors of the method, since the characteristic sickle shape of the rat sperm head (see Fig.8) makes it a peculiarly unsatisfactory object for cytophotometry.

Tumour-bearing liver. The effect of the carcinogen p-dimethylaminoazobenzene on the DNA content of the liver nuclei is shown in Fig.13. The tumour-bearing liver used in this experiment was taken from a rat which had been maintained on the carcinogenic diet for 14 weeks. Only part of the organ, which was extensively infiltrated with diffuse tumour nodules, was used for the isolation of nuclei for cytophotometry, the remainder being used for the estimation of the average DNAP content per nucleus by the chemical method used in the previous series of experiments. The mean DNA content of the 168 kidney nuclei measured cytophotometrically in this experiment is 360 units and of the 222 liver nuclei, 441 units. By the cytophotometric method, therefore, the ratio

$$\frac{\text{Mean DNA content of nuclei of tumour-bearing liver}}{\text{" " " " " " normal kidney}} = \frac{441}{360}$$

$$= 1.22.$$

The average DNAP content per nucleus found for the tumour-bearing liver by chemical analysis of nuclei isolated from

Figure 13.

Frequency histograms of deoxyribonucleic acid (DNA) contents of individual nuclei of rat kidney and tumour-bearing liver as estimated by the cytophotometric method described in Section 3.4. Two nuclei of the tumour-bearing liver with DNA contents of 3120 and 3160 arbitrary units are not shown.

Class interval = 40 units.

N.B. The arbitrary units used in this Figure are not identical with those used in Figures 9, 10, 11, 12, 14 or 15, or in Tables 64 or 66.

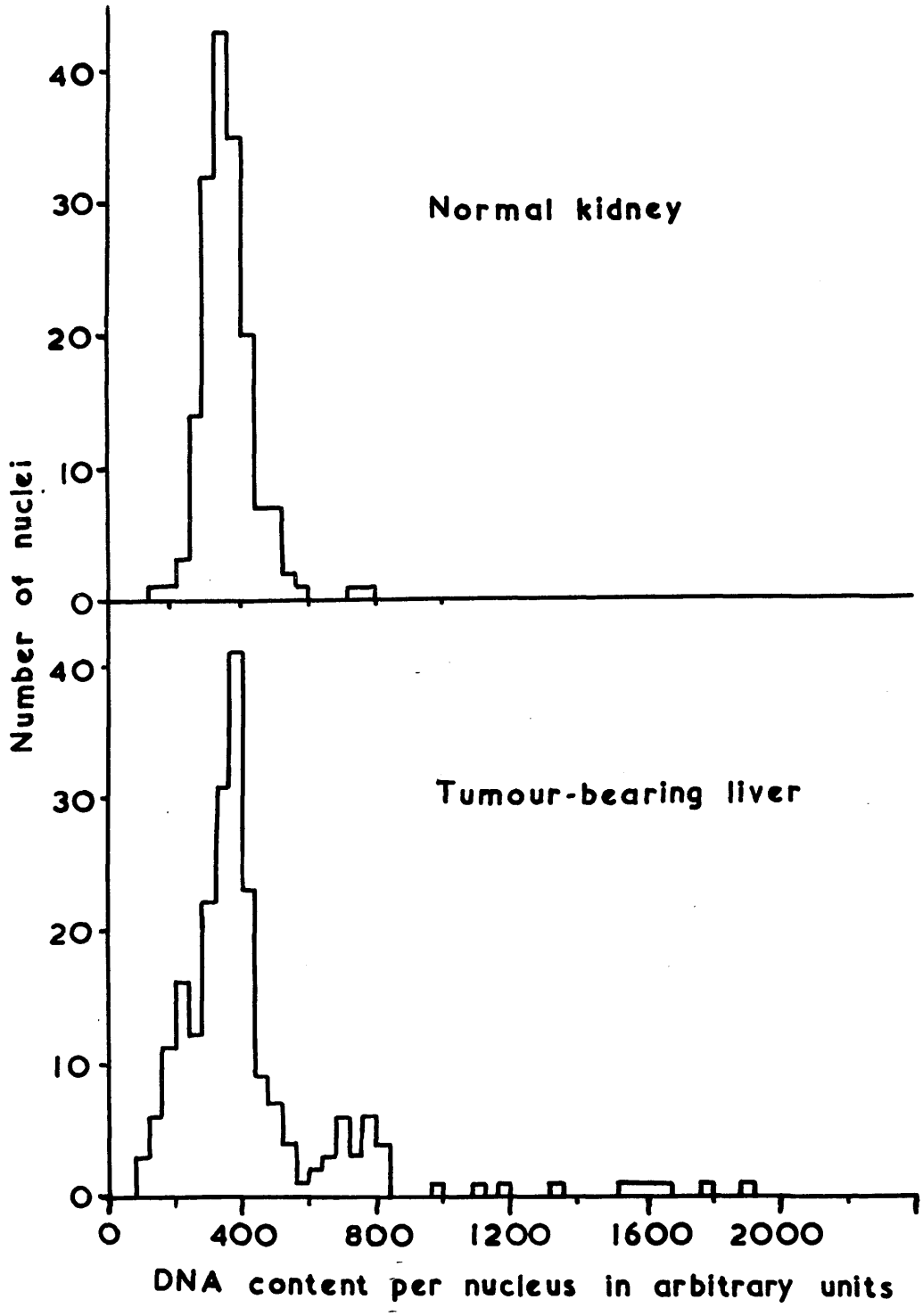
Normal kidney
Tumour-bearing liver
Number of nuclei
40
30
20
10
0
0
400
800
1200
1600
2000
DNA content per nucleus in arbitrary units

Figure 13.

the portion of this tissue not required for cytophotometry was 0.83 pg. The average DNAP content per nucleus for normal kidney has already been found by the chemical method to be about 0.65 pg. (see Table 20).

By the chemical method, therefore, the ratio

$$\frac{\text{Mean DNA content of nuclei of tumour-bearing liver}}{\text{" " " " " " normal kidney}} = \frac{0.83}{0.65}$$

$$= 1.28.$$

Again the agreement between the ratios found by the two methods is remarkably good.

Of the 168 kidney nuclei shown in Fig.13, 166 form a single peak extending from 160 to 500 units (mean = 358 units) and belong to Class I. The other 2 nuclei with DNA contents of 720 to 800 units belong presumably to Class II. The nuclei of the cancerous liver appear to fall into the same three classes as the nuclei of normal liver. If, as in the case of normal liver and pancreas, the upper limit of the Class I kidney nuclei (in the present experiment, 600 units) can be taken as the boundary between the Class I and Class II nuclei in the liver, the 222 liver nuclei measured may be divided into

186 Class I nuclei, i.e., 83.7% of the whole sample, with a DNA content of 80 to 600 units, mean = 336 units;

24 Class II nuclei, i.e., 10.8% of the whole sample,

with a DNA content of 600 to 840 units, mean = 733 units;

10 Class III nuclei, i.e., 4.5% of the whole sample, with a DNA content of 960 - 1920 units, mean = 1468 units;

and 2 nuclei, i.e., 0.9% of the whole sample, with a DNA content of 3120 - 3160 units, mean = 3140 units, which presumably constitute a Class IV.

Once again, the mean DNA contents for Classes I, II, III and IV are approximately in the geometrical progression 1 : 2:: 4 : 8 and the mean DNA content for Class I liver nuclei is roughly the same as for Class I kidney nuclei.

Since the arbitrary units used in Figs.9 and 13 are not necessarily of the same magnitude, a direct comparison of the numerical results obtained for normal and cancerous liver is not possible. If, however, the general patterns of the results shown in Figs.9 and 13 are compared, it becomes apparent that the most striking effect of the carginogen has been to increase very considerably the proportion of Class I nuclei in the liver (from 58.7% to 83.7%) and simultaneously to decrease the proportion of tetraploid nuclei from 39.9% to 10.8%. To some extent at least, it would seem reasonable to interpret these changes as indicating the "dilution" of the nuclei of the normal liver by

diploid tumour nuclei. The possibility that other factors may also be involved (e.g., destruction of normal liver tissue due to compression by growing tumour nodules) must not, however, be overlooked.

Regenerating liver. The DNA contents of nuclei isolated from a portion of the remaining fragment of liver 48 hours after partial hepatectomy (i.e., during the phase of very rapid growth) are shown in Fig.14. A second portion of the same fragment of liver was used to obtain an estimate of average DNAP content per nucleus by the chemical technique used in the previous series of experiments. The mean DNA content of the 371 nuclei of regenerating liver which were measured cytophotometrically is 1053 units and the corresponding figure for the 139 kidney nuclei which served as controls is 560 units. By the cytophotometric method, therefore, the ratio

$$\frac{\text{Mean DNA content of nuclei of regenerating liver}}{\text{" " " " " " normal kidney}} = \frac{1053}{560}$$

$$= 1.88.$$

The average DNAP content per nucleus for the regenerating liver as determined by chemical analysis of nuclei isolated from the portion of this tissue not required for cytophotometry was 1.26 pg. This figure is in good agreement with the results previously obtained for this tissue by the

Figure 14.

Frequency histograms of the deoxyribonucleic acid (DNA) contents of nuclei of rat kidney and regenerating liver as estimated by the cytophotometric method described in Section 3.4.

Class interval = 80 arbitrary units.

N.B. The arbitrary units used in this Figure are not identical with those used in Figures 9, 10, 11, 12, 13 or 15, or in Tables 64 or 66.

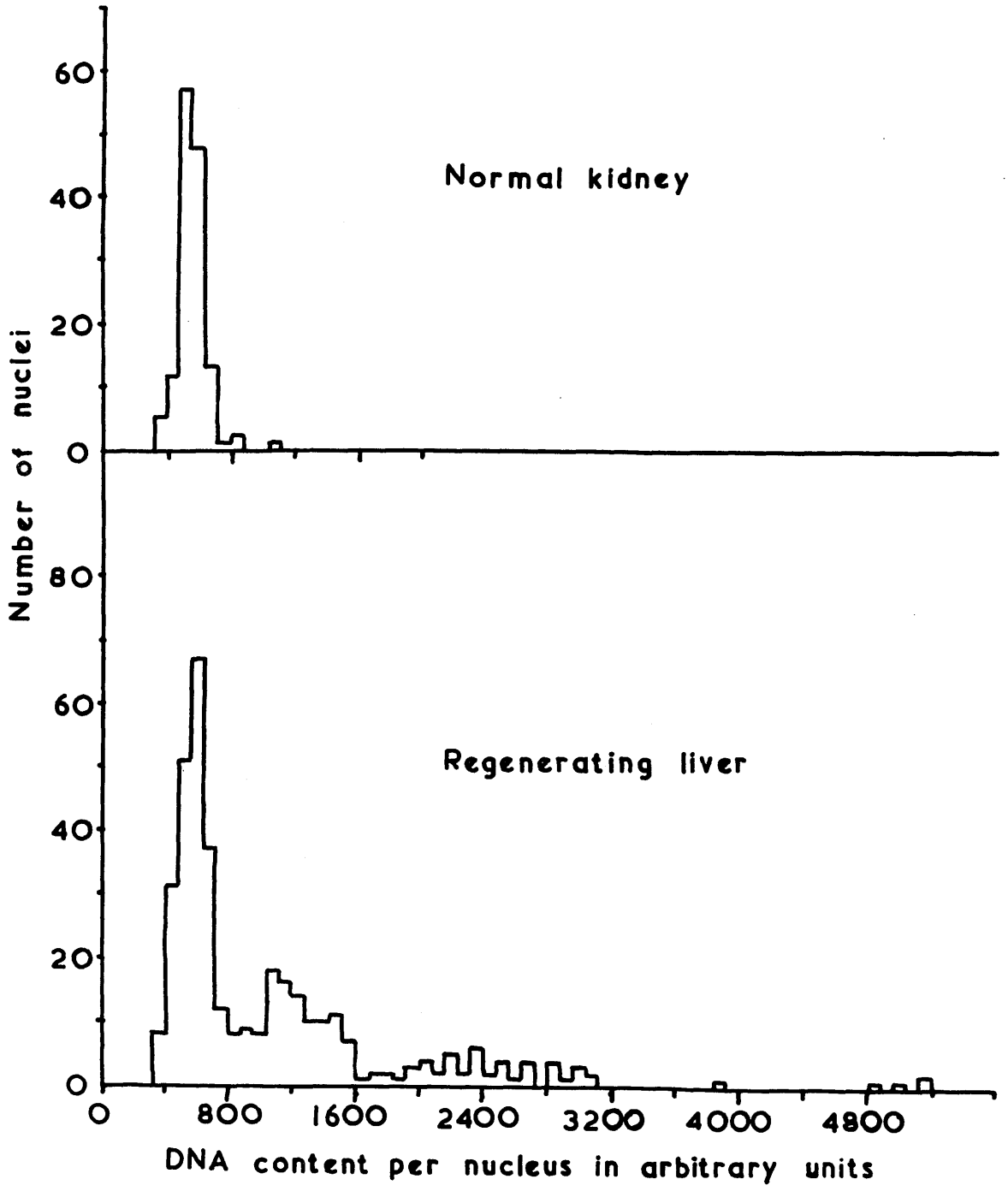
Normal kidney
Regenerating liver
Number of nuclei
60
40
20
0
80
60
40
20
0
0
800
1600
2400
3200
4000
4800
DNA content per nucleus in arbitrary units

Figure 14.

chemical method (see Table 25). The average DNAP content per nucleus for normal kidney has already been found by the chemical method to be about 0.65 pg. By the chemical method, therefore, the ratio

$$\frac{\text{Mean DNA content of nuclei of regenerating liver}}{\text{" " " " " " normal kidney}} = \frac{1.256}{0.65}$$

$$= 1.93,$$

which is in remarkably good agreement with the ratio found by the cytophotometric method.

From Fig.14 it will be seen that 138 of the 139 kidney nuclei measured form the usual symmetrical and fairly compact Class I peak extending from 320 to 880 units with a mean of 557 units. The remaining nucleus, containing 1080 units of DNA, presumably belong to Class II. The nuclei of the regenerating liver fall into the same four Classes as those of the tumour bearing liver. Taking the upper limit of the Class I kidney nuclei as the boundary between Class I and Class II nuclei in the liver, the 371 liver nuclei may be divided into

214 Class I nuclei, i.e., 57.7% of the whole sample, containing 320 to 880 units of DNA, mean = 580 units;

103 Class II nuclei, i.e., 27.7% of the whole sample, containing 880 to 1600 units of DNA, mean = 1224 units;

49 Class III nuclei, i.e., 13.2% of the whole sample, containing 1600 to 3120 units of DNA, mean = 2373 units;

and 5 Class IV nuclei, i.e., 1.35% of the whole sample, containing 3840 to 5200 units of DNA, mean = 4808 units.

Once again the mean DNA contents for Classes I, II, III and IV are approximately in the ratio 1 : 2 : 4 : 8 and the mean DNA content for Class I liver nuclei is approximately the same as for Class I kidney nuclei.

If the general pattern of these results is compared with that shown in Fig.9, it is apparent that the regenerating liver contains the same three Classes of nuclei as the normal organ in somewhat different proportions. In particular, the percentage of Class II nuclei has fallen from 39.9 to 27.7 and there has been a corresponding increase in the percentage of Class III nuclei from 1.46 to 13.2. At the same time a few nuclei which appear to belong to Class IV have emerged. Presumably these changes are responsible for the marked but transient increase in average DNAP content per nucleus during the first four days following partial hepatectomy which was observed in the previous series of experiments.

Hitherto the terms Class II, Class III and Class IV have been used as though they were synonymous with tetraploid, octoploid and 16-ploid, respectively. This practice may not, however, be entirely justified since if Swift (1950a) and his associates (see Section 2.4) are correct in their theory that a nucleus about to undergo mitosis doubles its content of DNA, it follows that, for example, a Class II nucleus may be either a resting interphase tetraploid nucleus or a diploid nucleus about to enter prophase. In view of the very high mitotic rate in regenerating liver (see Section 2.4) it seems most probable that a large proportion of the Class III and Class IV nuclei in this tissue are, in fact, tetraploids and octoploids which have doubled their DNA content in preparation for mitosis rather than resting interphase octoploids and 16-ploids.

Variation between nuclei of a single class. One of the somewhat surprising features of the results obtained in the present series of experiments was the wide scatter of values obtained for different nuclei belonging to the same Class. The frequency distribution for kidney nuclei shown in Fig.9 may be taken as an example. Even if the two nuclei containing 960 - 1040 units of DNA are excluded as belonging to Class II, the remainder, all of which belong presumably to Class I, give figures of anything from 280

to 720 units of DNA with a mean of 500 units and a standard deviation of 87 units. The coefficient of variation for this group, therefore, is $^{87}/_{500} \times 100 = 17.4\%$. This wide scatter might be interpreted in two ways. It might be assumed that the cytophotometric method used was fairly accurate and that the observed scatter of results reflected a true biological variation in DNA content between different nuclei of the same Class. Alternatively, it might be assumed that all the nuclei measured had exactly the same content of DNA and that the observed scatter was due to errors inherent in the method of measurement. Clearly, it was of considerable importance to determine which of these explanations was correct.

Accordingly, a smear of normal kidney nuclei was photographed in the usual way. One of the frames of the negative so obtained was selected for measurement. Of the 25 nuclei which it contained one was rejected as being apparently damaged and the remaining 24 were numbered. The DNA contents of these nuclei were estimated in the usual way 4 times, in random order each time. The figures obtained (shown in Table 64) were submitted to analysis of variance (Snedecor, 1946; Brownlee, 1948). The results of this analysis (Table 65) showed a significant difference between nuclei and the corresponding coefficient of variation was

Table 64.

Statistical experiment to determine whether there is any significant difference in DNA content between individual rat kidney nuclei of the same Class.

DNA contents of 24 kidney nuclei measured 4 times, in random order each time.

Nucleus No.	DNA content in arbitrary units*			
	1st estimate	2nd estimate	3rd estimate	4th estimate
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	363	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.

*N.B. The arbitrary units in which these estimates are expressed are not identical with those used in Figs.9, 10, 11, 12, 13, 14 or 15, or in Table 66.

Grand mean of the 24 nuclei = 365.3 arbitrary units.

Table 65.

Statistical experiment to determine whether there is any significant difference in DNA content between individual rat kidney nuclei of the same Class.

Analysis of variance of data presented in Table 64.

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio "F"
Between nuclei	23	54,537	2371.2	2.09
Between estimates	3	12,197	4065.7	
Residual error	69	78,197	1133.3	
Total	95	144,931		

For $n_1 = 24$, $n_2 = 70$, $F = 2.07$ 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 $\frac{2371.2 - 1133.3}{4} = 309.5$. Since the grand mean for the 24 nuclei = 365.3 (Table 64), the coefficient of variation between nuclei = 309.5 / 365.3 = 4.8%.

Figure 15.

Frequency histogram of mean values for deoxyribonucleic acid (DNA) content of rat liver nuclei shown in Table 66.

Class interval = 80 units.

N.B. The arbitrary units used in this Figure are not identical with those used in Figures 9, 10, 11, 12, 13 or 14 or in Table 64.

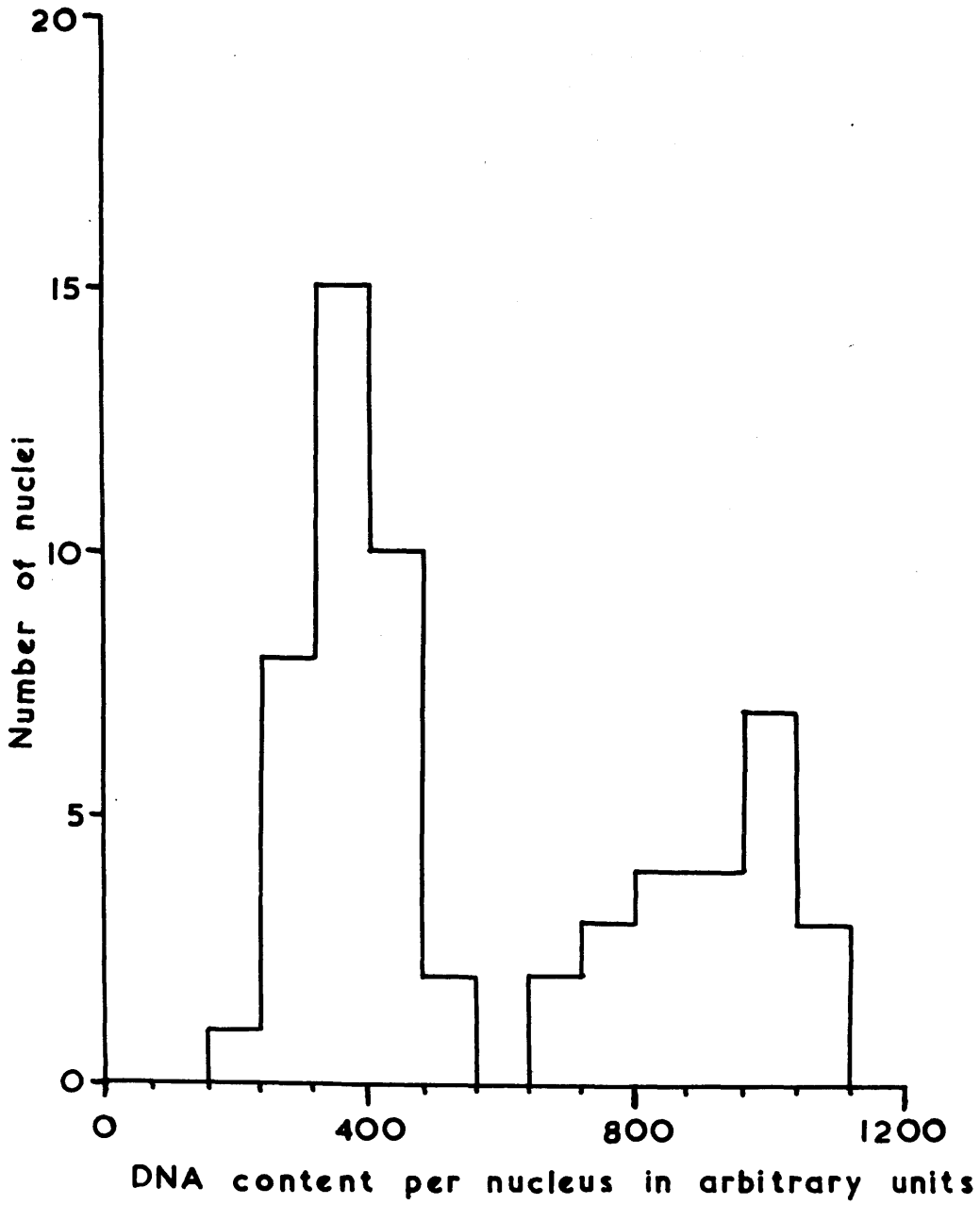
20
15
10
5
0
Number of nuclei
0
400
800
1200
DNA content per nucleus in arbitrary units

Figure 15.

Table 66.

Statistical experiment to determine whether there is any significant difference in DNA content between individual rat liver nuclei belonging to the same Class.

DNA contents of 59 liver nuclei estimated twice, in random order each time.

Note:- When the mean values of the two measurements made on each of the 59 nuclei were plotted in the form of a frequency histogram (Fig.15), it became clear that the nuclei fell into two discrete groups:

(i) 36 nuclei for which mean values of 160 to 560 arbitrary units were obtained and which all presumably belonged to Class I; and
(ii) 23 nuclei for which mean values of 640 to 1120 arbitrary units were obtained and which all presumably belonged to Class II.

In the present Table the figures obtained for the two groups are listed separately.

Class I nuclei

Nucleus No.	DNA content in arbitrary units		
	1st estimate	2nd estimate	Mean
1/3	460	462	461
1/4	396	522	459
1/5	430	510	470
1/7	485	352	419
1/10	264	280	272
1/12	309	281	295
1/14	374	297	336
1/15	429	277	353
1/16	388	371	380
2/1	422	424	423
2/3	435	477	456
2/4	539	551	545
2/6	275	271	273
2/7	340	367	354
2/8	205	234	220
2/9	482	506	494
2/12	315	399	357
2/13	345	354	350
2/15	315	346	331
2/16	531	409	470
2/17	347	436	392

Table 66 (Contd.)

Class I nuclei continued

Nucleus No.	DNA content in arbitrary units		
	1st estimate	2nd estimate	Mean
2/20	375	357	366
2/21	424	413	419
3/1	304	286	295
3/3	306	292	299
3/5	276	281	279
3/6	292	283	288
3/7	413	423	418
3/8	357	355	356
3/10	373	316	345
3/12	404	297	351
3/13	320	405	363
3/15	314	255	285
3/16	348	367	358
3/18	409	372	391
3/19	413	420	417.

Grand mean of the 36 Class I nuclei
= 370.30 arbitrary units.

Class II nuclei

1/1	834	600	717
1/2	794	875	835
1/6	924	910	917
1/9	846	831	839
1/11	1042	1037	1040
1/13	964	945	955
1/17	939	1015	977
1/18	835	882	859
1/19	1118	971	1045
2/2	995	974	985
2/5	889	680	785
2/10	1042	977	1010
2/14	952	1141	1047
2/18	788	685	737
2/19	1090	1053	1072
2/22	940	885	913
2/23	746	913	830
3/2	687	720	704

Table 66 (Contd.)

Class II nuclei continued.

Nucleus No.	DNA content in arbitrary units		
	1st estimate	2nd estimate	Mean
3/4	695	836	766
3/9	1019	1044	1032
3/11	1045	996	1021
3/14	785	1008	897
3/17	898	1040	969

Grand mean of the 23 Class II nuclei
= 910.5 arbitrary units.

N.B. The arbitrary units used throughout this Table (and in Fig.15) are not identical with those used in Figs.9, 10, 11, 12, 13 or 14, or in Table 64.

Table 67.

Analysis of variance to determine whether there is any significant difference in DNA content between the 36 Class I liver nuclei in Table 66.

Source of variation	Degrees 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 $\frac{10,763 - 1,899}{2} = 4432$. Since the grand mean for the 36 nuclei = 370.3 (Table 66), the coefficient of variation between nuclei = 4432 / 370.3 = 18%.

Table 68.

Analysis of variance to determine whether there is any significant difference in DNA content between the 23 Class II nuclei in Table 66.

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 error	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 $\frac{27158 - 7081}{2} = 10039$. Since the grand mean for the 23 nuclei = 910.5 (Table 66), the coefficient of variation between nuclei = 10039 / 910.5 = 11%.

estimated to be 4.8% of the mean DNA content of the 24 nuclei measured. The real variation in DNA content from one Class I nucleus to another may, in fact, be less than this estimate, since some of the errors of the method cannot be eliminated by replicating measurements, e.g., the error introduced by the fact that, although the method is theoretically valid only for spherical or ellipsoidal nuclei in which the absorbing material is evenly distributed within the nucleus (Frazer & Davidson, 1953; see also Ornstein, 1952), it is being applied in the present instance to nuclei which only approximate, in varying degrees, to these theoretical requirements.

A similar statistical test was applied to Class I and Class II liver nuclei. Three adjacent frames of liver nuclei were selected from the negative from which the data shown in Fig.9 were obtained. The 59 nuclei in these frames were numbered and their DNA content measured twice in the usual way, in random order each time. The individual figures obtained are shown in Table 66. The means of the two figures obtained for each nucleus are plotted in the form of a frequency histogram in Fig.15. They fall into two distinct peaks extending from 160 to 560 units and from 680 to 1080 units. It was presumed that all the nuclei of the first group belonged to Class I and all those of the

second group to Class II. Accordingly, the individual figures obtained for each group were submitted to analysis of variance as in the case of kidney nuclei (see Tables 67 and 68). By this means it was established that in both groups there was a significant variation in DNA content between individual nuclei. The magnitude of the corresponding coefficients of variation was estimated to be 18% of the mean DNA content for Class I nuclei and 11% of the mean for Class II nuclei. Although these figures are considerably larger than that found for the kidney nuclei this need not necessarily mean that the variation in DNA content between individual nuclei within a single Class is greater in liver than in kidney but may merely be a reflection of the fact that the distribution of the Feulgen stain was generally less uniform within the individual liver nucleus than within the individual kidney nucleus.

3.6 Discussion.

It has been shown in the preceding section that wherever the average DNA content of the nuclei of a tissue could be estimated by both chemical analysis of counted suspensions of isolated nuclei and by the cytophotometric method of Frazer & Davidson (1953), the agreement between the results of the two methods was remarkably good. It seems therefore justifiable to assume that the latter technique is a fairly

reliable means of estimating, at least approximately, the relative DNA content of individual nuclei.

The results which have been obtained by its use in the present series of experiments might be summarised as follows. All the nuclei of the six somatic tissues investigated (normal kidney, liver, intestine and pancreas, cancerous liver and liver regenerating after partial hepatectomy) fall into four classes with respect to their content of DNA. Class I nuclei, which contain approximately twice as much DNA as spermatozoa, are the most common class in all six tissues. Class II nuclei, which contain approximately twice as much DNA as Class I, are numerous in normal liver and pancreas, in cancerous liver and in regenerating liver. A few of the nuclei of normal kidney and intestine also appear to belong to this Class. Class III nuclei, containing approximately twice as much DNA as Class II and four times as much as Class I, were found occasionally in normal liver and cancerous liver, and more frequently in regenerating liver. A few of the nuclei in regenerating liver and in cancerous liver contain twice as much DNA as Class III (i.e., eight times as much as Class I) and may be regarded as constituting a Class IV. The errors of the method do not allow of an accurate estimate being made of the differences in DNA content between individual nuclei within the same tissue and belonging to the same class. It seems

probable, however, that the coefficient of variation for these differences does not greatly exceed 15% and may even be less than 5%. Evidence has been presented suggesting that in normal liver and pancreas Classes II and III probably represent interphase tetraploid and octoploid nuclei respectively. On the other hand, it seems probable that many of the Class III and Class IV nuclei found in regenerating liver may well be respectively tetraploid and octoploid nuclei which have doubled their DNA content preparatory to undergoing mitosis (see Section 2.4).

These results clearly support the hypothesis that the DNA content per set of chromosomes is at least approximately constant for the nuclei of a single species. In this respect they are in agreement with those obtained by most other workers who have used the cytophotometric technique to estimate the DNA content of the nuclei of rat and mouse tissues (Ris & Mirsky, 1949a; Swift, 1950a; Leuchtenberger, Vendrely & Vendrely, 1951; Leuchtenberger, Leuchtenberger, Vendrely & Vendrely, 1952; Alfert, unpublished results cited by Pollister, 1952b; Frazer & Davidson, 1953). Moreover, inasmuch as they indicate that polyploid nuclei containing 2, 4 or even 8 times as much DNA as normal diploid nuclei may not be uncommon in the various tissues of the rat, they suggest an explanation for the observation of Cunningham

et al. (1950) that in the rat the average DNA content per nucleus is anomalously high in the external orbital gland as well as in the liver (see Table 18), particularly since there is evidence of a purely histological nature for the occurrence of polyploidy in this tissue (Teir, 1944). Similarly Leuchtenberger, Klein & Klein (1952a, b) have shown that in the mouse the DNA content of nuclei of Ehrlich ascites tumours measured cytophotometrically is twice that found in normal diploid nuclei and have demonstrated by means of chromosome counts that this is due to polyploidy. It seems probable that the observation of Petermann & Schneider (1951) that in the same species the average DNA content per nucleus, determined chemically, is higher in leukaemic spleen than in the normal organ may also be explained in terms of polyploidy (Mizen & Petermann, 1952). It would appear, therefore, that the variations in average DNA content per nucleus from tissue to tissue which are found within a single species are, in general, due to the presence of varying proportions of polyploid nuclei. At the same time it may be noted that Mirsky & Ris (1951) have now withdrawn their claim (see Section 1.11 and Table 17) that the average DNA content of the somatic nuclei of beef tissues is more than twice that of bull sperm. The original reasons for believing that the Boivin-Vendrely hypothesis might not be

valid for mammalian species (see Section 1.11) have thus lost their force.

There remains, however, the claim of Pasteels & Lison (1950a), already cited in Section 3.1, that, although there are three classes of nuclei in rat liver and pancreas, the characteristic DNA contents of which are in the approximate ratio 1 : 2 : 4, the characteristic DNA content for each class is about 30% below the theoretical diploid or tetraploid or octoploid value, as determined by measurements on the nuclei of other tissues. Observing that no other group of workers were able to confirm their findings on this point, Pasteels & Lison (1953) have reinvestigated the question for themselves by carrying out measurements on a further three rats. In these animals they find, as all other workers who have made similar measurements have found, that the average DNA content of the Class II nuclei of the liver is not significantly different from the theoretical tetraploid amount (as estimated, in this case, from measurements on primary spermatocytes, which both Pasteels & Lison, 1950a, and Swift, 1950a, have shown to have twice the DNA content of the normal diploid nuclei). At the same time they have repeated their measurements on the sections, taken from a single animal, which they used in their original experiments, and have again reported that in this animal the Class II

liver nuclei contained only about 70% of the theoretical tetraploid amount. From these observations they conclude that the mean DNA contents of the three classes of liver nuclei vary from one animal to another and that the constancy of the DNA content per set of chromosomes must not be therefore regarded "à l'instar d'un dogme". This seems a very sweeping generalisation to make on the basis of an examination of no more than four animals, of which three gave figures which are in perfect accordance with the "dogma", while the anomalous results obtained from the fourth, although first published almost three years ago, have never been confirmed for another animal either by Pasteels & Lison or by anyone else. Under these circumstances it seems not unreasonable to assume, until Pasteels & Lison produce evidence to the contrary, that the results obtained from this animal were due to some artefact in preparing the tissues for cytophotometry.

Mention must also be made of the cytophotometric results recently reported by Sibatani, Fukuda, Matsuda & Naora (1952). These workers find that the diploid nuclei of rat kidney, adrenal, pancreas and cerebellum all contain approximately the same amount of DNA while the tetraploid nuclei of liver contain twice this amount. On the other hand the nuclei of oesophageal epithelium are reported as containing less than

the diploid amount of DNA, and the primary spermatocytes (contrary to both Pasteels & Lison, 1950 and Swift, 1950a) as containing more than the tetraploid amount. While these observations have been published only as a brief note and cannot therefore be critically assessed, it may well be that the two apparent exceptions to the Boivin-Vendrely hypothesis may be less significant than might be thought at first sight. Histological examination of the epithelium of the rat oesophagus shows it to be of the stratified squamous type in which cells are being continuously desquamated from the surface and being replaced from below. It may well be that the low average DNA content found for the nuclei of such cells is simply a reflection of the pyknosis and consequent loss of DNA (Leuchtenberger, 1950) which occurs as the cell, becoming more and more flattened and scale-like in appearance, approaches the outer surface of the epithelium. On the other hand, the anomalously high figure obtained for primary spermatocytes may be related to the fact that they were apparently measured at the leptotene stage since it is doubtful whether at this point in the meiotic process the DNA of the spermatocyte nucleus would be sufficiently evenly distributed to allow accurate cytophotometric measurements to be made (Ornstein, 1952).

It seems, therefore, that on the basis of the present

series of experiments, of the previous chemical investigation (Section 2.3) and of the observations reported by other workers, we may reasonably conclude that the DNA content per set of chromosomes is approximately constant for the resting interphase nuclei of the different tissues of the rat. Whether there may be small biological variations in DNA content per set of chromosomes between individual nuclei is still uncertain. The arguments originally put forward by Boivin, Vendrely & Vendrely (1948) suggest rather that the DNA content per nucleus (except in the case of polyploid or pyknotic or "pre-mitotic" nuclei) should be absolutely constant for any species - a sort of biological atomic weight. As more and more cytophotometric investigations have appeared to show that there is some variation in DNA content between individual nuclei of the same class and within the same tissue, the correctness of this "rigorous" view has come to be doubted (Leuchtenberger, Vendrely & Vendrely, 1951; Leuchtenberger & Schrader, 1952; Davidson, 1953). It should, however, be pointed out that, as has been shown in the preceding section, some at least of this apparent variation between nuclei is due to the errors inherent in the cytophotometric method. Clearly, therefore, a definite answer to the question of whether the DNA content per set of chromosomes in different nuclei is absolutely constant or only approximately

so will only be possible when more precise and reliable techniques for measuring the relative DNA content of individual nuclei have been devised.

Part IV.

General Discussion.

General Discussion.

4.1 The deoxyribonucleic acid content of the cell nucleus.

The results obtained in the present series of experiments by both chemical analysis of counted suspensions of isolated nuclei and cytophotometric measurements on individual nuclei indicate fairly conclusively that the DNA content per set of chromosomes is at least approximately constant for the resting interphase nuclei of the different tissues and organs of the rat. In view of the results previously reported by the Vendrelys (see Table 16), by Mirsky & Ris (see Table 17), by Davidson and his associates (see Table 19) and by Swift (see Section 3.1) it would seem reasonable to assume that this conclusion is valid also for other animal species. This assumption is supported by the results of a number of experiments of a rather miscellaneous character. Schrader & Leuchtenberger (1950), for example, have shown by the cytophotometric method that in the pentatomid insect Arvelius albopunctatus all spermatocyte nuclei have the same content of DNA although their volumes and protein contents vary quite widely. Similarly, Alfert (1951) has shown that, in the mouse, all the primary oöcyte nuclei on which satisfactory cytophotometric measurements could be made contained approximately the same amount of DNA in spite of the wide variation in their volumes. Davison & Osgood

(unpublished experiments cited by Mirsky & Ris, 1951) have shown, by gross chemical analysis of counted suspensions of cells, that in man the granulocytes and lymphocytes (two quite different cell types) both contain approximately twice as much DNA as the spermatozoa. Reisner & Korson (1951), using the cytophotometric technique, have found that the DNA content of human erythroblast nuclei is the same for normal and anaemic subjects and that, in the case of the latter, it is unaffected by treatment with vitamin B_{12}, folic acid or liver extract. Using the same method Alfert & Bern (1951) have studied the effects of oestrogen on the nuclei of the uterine glands of the rat and have reported that it produces a marked increase in nuclear volume and protein content but no change in DNA content. In a somewhat similar fashion Di Stefano, Bass, Diermeier & Tepperman (1952) have found that hypophysectomy and administration of growth hormone affect the RNA and protein content of rat liver nuclei but not their content of DNA. Finally, Moore (1952) has produced androgenetic haploid frog embryos by removing the maternal chromosomes from the fertilized egg and has shown cytophotometrically that the nuclei in the tissues of such embryos contain approximately half as much DNA as the corresponding nuclei of normal diploid embryos at the same stage of development.

On the other hand, in only one case has evidence been

produced for the occurrence in an animal species of a wide and irregular variation in the DNA content of the nuclei which is not apparently correlated with polyploidy, polyteny, mitosis or pyknosis. In the salivary gland of the snail Helix aspersa the nuclei of non-secreting cells have been shown to have a much higher content of DNA (as determined by cytophotometric measurements on Feulgen-stained preparations) than those of actively secreting cells (Leuchtenberger & Schrader, 1952). Since there appears to be an inverse relationship between the size and DNA content of the nucleus on the one hand and the amount of secretion visible in the cytoplasm on the other, it is suggested that the secretion may be derived from degradation products of DNA. The mechanism by which this postulated process might take place is completely obscure.

It appears, therefore, that the balance of the evidence published in the literature supports the view that the modified Boivin-Vendrely hypothesis is valid not only for the rat but for animal species in general, at least in the case of tissues which are not growing rapidly. The significance of results obtained from tissues which are growing rapidly is, for several reasons, much more difficult to assess.

1. The results obtained in the present investigation for liver regenerating after partial hepatectomy, as well as those previously reported by Swift (1950a) have indicated

that in such a tissue individual nuclei of the same ploidy cannot all be expected to have the same content of DNA, since some will be doubling their content of DNA preparatory to mitosis. The fact, therefore, that Moore (1952) found considerable variations in DNA content between individual nuclei in the tissues of frog embryos and especially in those of early embryos need not be taken as invalidating the modified Boivin-Vendrely hypothesis. It must, of course, be appreciated that, while the presence of such "premitotic" nuclei with an increased content of DNA can be detected fairly readily by the cytophotometric method, only in a tissue in which mitoses are exceptionally frequent will the proportion of such nuclei be so great as to produce a perceptible increase in the average DNA content per nucleus. For example, following partial hepatectomy in the rat the number of cells in the remaining fragment of liver doubles within 4 - 6 days (see Table 25). Yet during this phenomenally rapid hyperplasia the average DNA content per nucleus does not rise above 150% of the normally resting level (Table 25). It is hardly surprising, therefore, that Davidson et al. (1950) should have found the tissues of the chick embryo, in which growth is much less rapid than in regenerating rat liver, to have the same average DNA content per nucleus as the tissues of adult birds of the same

species (see Table 19), although subsequent cytophotometric experiments by Frazer & Davidson (1953) have suggested that a small proportion of the nuclei in chick embryo liver (in which polyploidy does not appear to occur) have a DNA content 30 - 100% above the normal resting diploid level.

2. It must be expected also that there will be a variation in average DNA content per nucleus from one tissue to another since different tissues will contain different proportions of nuclei preparing for mitosis. For example, Davidson, Leslie & White (1950, 1951) have demonstrated that in man the average DNA content of the leucocytes of the peripheral blood, a cell type in which polyploidy does not occur (Mandel, Metais & Cuny, 1950; Mandel, 1951), and which does not undergo mitosis, is approximately twice the DNA content of the spermatozoa, whereas the sternal bone marrow, a tissue in which haemopoiesis is active and mitoses are relatively numerous, has a DNA content per nucleus appreciably in excess of this amount. This phenomenon is probably also the cause of the variation in DNA content per nucleus between different tissues of the frog embryo which was noted by Moore (1952).

3. The average DNA content per nucleus in a growing tissue will depend to some extent also on external circumstances. Any factor, for example, which tends to inhibit

mitosis without affecting the synthesis of DNA will tend to cause nuclei which have doubled their DNA content in preparation for mitosis to accumulate, thus causing an increase in the average DNA content per nucleus for the tissue. It has already been suggested that this mechanism might be responsible for the increase in DNA content of the liver nuclei which according to Ely & Ross (1951a) and Lecomte & Smul (1952) is produced in young growing rats which have been fed a protein-free diet. It may equally well be responsible for the abnormally high values found by Davidson, Leslie & White (1950, 1951) for the DNA content per nucleus in the sternal marrow of human subjects suffering from megaloblastic anaemias (see also Menten, 1952; Menten & Willms, 1952a, b).

The most important evidence on the DNA content of the nuclei of rapidly growing tissues has, however, been obtained by the use of the cytophotometric technique. Alfert (1951) has used this method to study the process of cleavage in the mouse. In this species the male and female pronuclei (which are, of course,haploid) do not fuse at fertilization but instead lie side by side until the chromosomes in each condense and form a common metaphase plate, in preparation for the first cleavage division. If the conclusions drawn from the present series of experiments are generally valid,

it would be predicted that the haploid pronuclei preparing for the first cleavage division should have a DNA content intermediate between the theoretical haploid and diploid values, while the frequently dividing diploid nuclei of the early embryo should have a DNA content intermediate between the theoretical diploid and tetraploid values. Alfert's (1951) experimental results were in complete agreement with both these predictions.

A somewhat different relationship between cell division and DNA synthesis has been described by Pasteels & Lison (1951). These workers have found, using the cytophotometric technique, that during the early embryonic development (up to the pluteus stage) of the sea urchin Paracentrotus lividus, the nuclei always contain much more than twice the DNA content of the spermatozoa. Moreover, the DNA content of the nuclei is generally greatest in those parts of the embryo in which growth is most rapid. These observations might perhaps be interpreted as indicating that DNA is being synthesized more rapidly than it can be used for the formation of new chromosomes and is being, as it were, stored for use in future mitoses. In this connection it is of interest to recall that one of the early objections to the Boivin-Vendrely hypothesis was that in Arbacia the ovum appeared to have a DNA content enormously greater than that of the spermatozoon (Vendrely & Vendrely,

1949b; Schmidt et al., 1949). It was later argued that this result was probably due to the presence in the ovum of unknown compounds which interfered with the reactions by which DNA was estimated and that, for this reason, the estimated DNA content of this cell was fallaciously high (Vendrely, 1952; Mirsky & Ris, 1951). Some colour was given to this suggestion by the fact that in the fertilized eggs of the mouse (Alfert, 1951) and of Ascaris megalocephala (Mirsky & Ris, 1951) the male and female pronuclei appear to contain the same amount of DNA. New light has, however, recently been thrown on the problem by the work of Hoff-Jorgensen & Zeuthen (1952). By using a microbiological assay method these workers have been able to show that, in the frog, the unfertilized egg contains approximately 5,000 times as much "deoxyriboside" (i.e., DNA, deoxyribonucleotides and deoxyribonucleosides) as the spermatozoon. The greater part of this deoxyriboside appears to be located not in the nucleus but in the cytoplasm. Following fertilization, the total deoxyriboside content of the egg remains constant for nearly eighteen hours and does not begin to rise until the embryo has reached the blastula stage and contains several thousand cells. There is some evidence to suggest that in the sea-urchin also the early growth of the embryo may take place without any corresponding increase

in the total DNA content of the egg (Zeuthen, 1951). These observations suggest strongly that, in some species at least, the unfertilized egg contains two types of DNA: (1) "functional" DNA, i.e., the actual DNA of the chromosomes, which is presumably equal in amount to the DNA of the spermatozoon; and (2) preformed DNA (or DNA precursors not readily distinguishable, by the usual methods of estimation, from DNA itself) which is available for the duplication of chromosomes in the frequent mitoses of the early embryo and which will therefore allow the embryo to complete the early stages of development unhampered by the necessity of synthesizing its own requirements of DNA.

It is clear, therefore, that the results obtained from rapidly growing tissues and from eggs, which at first sight might appear to invalidate the modified Boivin-Vendrely hypothesis, are, in fact, perfectly consistent with it and, indeed, inasmuch as they indicate that mitosis does not take place until sufficient DNA has been accumulated to give both daughter nuclei their full resting complement of DNA, they provide fresh evidence of the cardinal importance of DNA in the life of the cell.

The validity of the modified Boivin-Vendrely hypothesis has not so far been demonstrated for any of the higher plants. It will be recalled that the wide and irregular

variations in DNA content between the nuclei of different tissues of Tradescantia which were reported by Schrader & Leuchtenberger (1949) constituted one of the early objections to the hypothesis. This question was re-investigated by Swift (1950b) who found that although in both Tradescantia and Zea mays the individual nuclei did indeed have different contents of DNA, these generally fell in the ratio 1 : 2 : 4 : 8 : 16 : 32. Swift suggested that this might be due to polyteny (reduplication of the chromonemata to give chromosomes with 2, 4, 8, etc., strands), the occurrence of which has been demonstrated in certain plant tissues (Lorz, 1947; Kaufmann, 1948) but which is not apparently known in the animal kingdom, except in certain special cases such as the salivary glands of Drosophila (D'Angelo, 1950). This seems quite a reasonable suggestion, since if the gene is a macromolecule of DNA it would be expected that a chromosome with X strands should contain X times as much DNA as the corresponding single-strand chromosome. Kurnick & Herskowitz (1952) have, in fact, shown that the DNA content of Drosophila salivary gland nuclei, in which the chromosomes are thought on cytological grounds to have about 1,000 strands, is approximately 1,000 times as great as that of the normal diploid nuclei of the same species. Swift was, moreover, able to show that the DNA content of the

nuclei which he studied was in some degree related to their ploidy since nuclei known on genetic grounds to be triploid contained 3, 6, 12 or 24 times as much as the microgamete nuclei. Unfortunately, since these observations were published, the situation has been somewhat complicated by Bryan's (1951) claim (which seems to be supported also by the data of Ogur, Erickson, Rosen, Sax & Holden, 1951) that in Tradescantia the microgamete contains not, as Swift believed, the haploid amount of DNA but the diploid amount. Clearly, until further information is available both on the occurrence of polyteny in plants and on the variations in DNA content between the nuclei of different plant tissues, the relationship between DNA and the gene in the higher plants will remain somewhat obscure.

The results obtained from micro-organisms, on the other hand, although not numerous, have been clear-cut and unambiguous. Caldwell & Hinshelwood (1950) have investigated, by chemical analysis of counted suspensions of cells, the DNA content per cell of the coliform organism Bacterium lactis aerogenes and found that it remains constant under a wide variety of different conditions. Heagy & Roper (1952) have shown, using the same method, that the conidia of haploid strains of Aspergillus nidulans contain approximately half as much DNA as the conidia of the corresponding diploid

strains. Finally, Ogur, Minkler, Lindegren & Lindegren (1952) have demonstrated, again by analysis of counted suspensions of cells, that the DNA contents per cell of four strains of yeast, known on genetical grounds to be haploid, diploid, triploid and tetraploid, are in the approximate ratio 1 : 2 : 3 : 4, whereas the mean cell weights and mean contents per cell of RNA and metaphosphate are in quite different ratios. It thus appears that the modified Boivin-Vendrely hypothesis is valid for micro-organisms.

In summary, it seems very probable that the theory that the DNA content per set of chromosomes is at least approximately constant for the resting interphase nuclei of any given species is generally valid for the higher animals and for micro-organisms. Deviations from the theory are found in the higher plants but there is reason to believe that this may be due to the occurrence of polytene chromosomes. These observations would seem to be most easily explained by assuming that in any given organism each gene is associated with a definite amount of DNA which is constant in all the nuclei of that organism.

4.2 Deoxyribonucleic acid and the gene.

The principal significance of the results of the present series of experiments lies in their bearing on the validity of the theory, implicit in the original arguments

of Boivin, Vendrely & Vendrely (1948), that the genes are essentially macromolecules of DNA and that this is the only function which DNA serves. This theory is based on the belief that the directed mutations of bacteria (see Section 1.10) can be most satisfactorily explained as being due to genes (or at least "genetic material") derived from killed donor cells penetrating the nuclei of living recipient cells and producing a permanent change in the hereditary character of the latter. Since the experiments of Avery and his colleagues and of Boivin (see Section 1.10) seem to show fairly conclusively that the transforming factors which produce such directed mutations consist of highly polymerized DNA, it would appear to follow that in bacteria the material of the genes must also be DNA. This argument has lost none of its cogency since it was first propounded by Boivin (1947). On the contrary, a detailed study by Alexander & Leidy (1950, 1951) has demonstrated that Haemophilus influenzae can undergo directed mutations similar to those previously produced in pneumococci and Escherichia coli. The transforming factor responsible for these mutations has subsequently been shown by Zamenhof, Leidy, Alexander, Fitzgerald & Chargaff (1952) to be a highly polymerised DNA. A development of possibly even greater significance is the demonstration by Hotchkiss (1951) that penicillin-sensitive

pneumococci can acquire resistance to this antibiotic by a directed mutation under the influence of a transforming factor derived from penicillin-resistant cells of the same species. The active principle of this transforming factor has also been isolated as a highly polymerised DNA only slightly contaminated with protein. More recently Alexander & Leidy (1953) have shown that streptomycin resistance may be induced in sensitive H. influenzae by DNA fractions obtained from resistant cultures of the same species. It seems, therefore, highly probable that, in bacteria at least, some genes are either themselves macromolecules of DNA, or, alternatively, require the presence of specific DNA molecules for their action. There is also the third possibility that the active principle of the transforming factors may be not DNA but some trace of contaminating protein which has not been removed in the course of purification. This view cannot be regarded as absolutely untenable but, as Avery et al. (1944), Zamenhof et al. (1952) and Austrian (1952) have shown, it is so unlikely to be correct that we may reasonably exclude it from consideration until some positive evidence is brought forward to support it.

If it is accepted that DNA is closely concerned with the functioning of the genes in bacteria, it may reasonably be assumed that it plays a similar rôle in the nuclei of the higher plants and animals. Most of the available

evidence is consistent with this assumption. On the basis of the results obtained by cytochemical techniques (Section 1.8) and by analysis of isolated chromatin threads (Table 12), it seems fairly certain that, in general, the DNA of the nucleus is localized on the chromosomes. The correctness of this view has indeed been denied by Stedman & Stedman (1943a, b, 1947a, b) who claim that chromosomin, the non-histone protein which they have obtained from isolated nuclei (see Section 1.5), is the principal, if not the sole, constituent of the chromosomes and that DNA and histone occur chiefly in the nuclear sap of the interphase nucleus and in the spindle of the dividing nucleus. No experimental evidence has been produced in support of this view, which assumes, of course,

(a) that the chromatin threads isolated by Mirsky and others (see Section 1.4), which have been shown by chemical analysis to contain DNA (Mirsky, 1947; see also Table 12), are not isolated interphase chromosomes,

(b) that the observation of Brown, Callan & Leaf (1950) that the nuclear sap of Triturus oöcytes contains non-histone protein is not valid for cell nuclei in general, and

(c) that the Stedmans' theory of the mechanism of the Feulgen reaction is correct, i.e., that fuchsin-

> sulphurous acid combines with soluble hydrolysis products of DNA to give a soluble purple dye which is adsorbed by chromosomin, and therefore that chromosomes give a positive Feulgen reaction not because they themselves contain DNA but because they contain chromosomin and are in close proximity to the DNA of the nuclear sap.

It has already been argued that this interpretation of the mechanism of the Feulgen reaction is probably invalid (Section 3.3). But even if its validity is accepted, it by no means follows that DNA is absent from the chromosomes and is confined to the nuclear sap. On the contrary Chu & Pai (1945) have shown that interphase chromosomes mechanically dissected out of the nuclei of Drosophila salivary glands and washed free of nuclear sap still give a positive Feulgen reaction, thus indicating that they must contain at least some of the DNA of the nucleus. The mere presence of DNA in the chromosomes does not by itself indicate that it is necessarily concerned with the functioning of the gene. The fact, however, that some of the so-called "mutagens" used by geneticists to increase the frequency of spontaneous mutations (e.g., irradiation with X-rays, treatment with mustard gas) are capable also of depolymerizing purified DNA in vitro strongly suggests that this may well be the case

(Chanutin & Gjessing, 1946; Sparrow & Rosenfeld, 1946; Taylor, Greenstein & Hollaender, 1947, 1948; Butler, 1949; Scholes, Stein & Weiss, 1949; Butler & Smith, 1950; Butler & Conway, 1950; Conway, Gilbert & Butler, 1950; Scholes & Weiss, 1952).

These observations, it will be noted, indicate that some at least of the DNA of the nucleus may be closely associated with the genes. They do not exclude the possibility that another fraction of the nuclear DNA might perform some quite different function. It is in this connection that the results of the present series of experiments are of particular significance. Let us suppose that the DNA of the nucleus does, in fact, consist of two or more fractions only one of which is closely associated with the genes. It may reasonably be assumed that the amount of "gene DNA" per set of chromosomes will be constant for all the resting interphase nuclei of an organism. On the other hand, it seems almost certain that the amount of "non-gene DNA" per set of chromosomes will vary from one cell type to another just as, for example, the enzyme content of the nucleus varies from one cell type to another (see Section 1.6). It seems equally probable that the amount of such "non-gene DNA" in a given resting interphase nucleus will depend on the physiological state of the organism. Clearly, if a

relatively large proportion of the DNA of the nucleus is in the "non-gene" fraction there will be considerable differences in total DNA content per set of chromosomes between the nuclei of different cell types, and the average DNA content of the nuclei of any given tissue will vary depending on the physiological state of the organism. But the results of the present series of experiments have indicated that in the resting interphase nuclei of the rat the DNA content per set of chromosomes is probably constant or variable only within narrow limits and that in at least one tissue (liver) the average DNA content of the nuclei is apparently unaffected by a wide variety of changes in the physiological condition of the animal. In this species, therefore, it may be presumed that all, or almost all, the DNA of the nucleus is closely associated with the genes. In the preceding section arguments were presented in favour of assuming that the average DNA content per set of chromosomes is constant not only for all animal species but also for micro-organisms and, if allowance is made for polyteny, for the higher plants. If these arguments are accepted it would follow that in all types of organisms the DNA of the nucleus should be regarded as being entirely and exclusively concerned with the functioning of the genes. If this conclusion is correct, it clearly marks a considerable

advance in our understanding of the biochemistry of the nucleus.

The precise nature of the postulated relationship between DNA and the gene has not yet been established. Boivin (1947), as has already been indicated, seems to have conceived of the gene itself as being essentially a macromolecule of DNA. This is certainly the simplest theory by which the observed facts can be explained and there cannot be said to be any definite evidence against it. The results of the investigations of nucleic acid composition carried out during the last six years have generally supported the view that DNA (and RNA) molecules may possess the same high degree of specificity as protein molecules (see Section 1.2). Moreover, the results of the most recent X-ray studies appear to indicate that the DNA molecule may have a structure of considerable geometrical complexity (Pauling & Corey, 1953a, b; Watson & Crick, 1953; Wilkins, Stokes & Wilson, 1953; Franklin & Gosling, 1953). The hypothesis that the gene is a macromolecule of DNA need not, therefore, be rejected on the ground that the DNA molecule is too simple to serve so complex a function.

The real difficulty in accepting the view that the gene consists entirely of DNA lies in trying to explain how the number and sequence of the nucleotides in a molecule of DNA can in any way influence the metabolism of the cell.

Dounce (1952b) has recently tried to solve this problem by postulating the existence of a series of enzymes by means of which a polypeptide chain might be synthesized alongside a nucleic acid molecule, the sequence of amino-acids in the former being determined by the sequence of nucleotides in the latter. In this way a particular nucleic acid molecule might catalyse (or "direct") the production of a large number of identical protein molecules. At the moment, unfortunately, this ingenious theory is purely speculative, none of the postulated enzymes having so far been shown to exist. Judgment on its validity must therefore be reserved until further evidence is available.

It may be that one reason for the general reluctance to believe that the gene consists entirely of DNA is that, prima facie, the proteins with their much more varied and intricate structure and their capacity, in certain cases, for performing highly specialised functions (e.g., myosin, cytochrome, pepsin, collagen, insulin) would seem much more likely to form the material of the gene than DNA. Moreover the generally accepted theory that in the production of antibodies the antigen molecules act as templates on which antibody molecules are formed (Boyd, 1947; Pauling, 1948; Haurowitz, 1949, 1952) would seem to provide a possible model of the mechanism by which a protein gene might direct the synthesis of a large number of identical protein

molecules. If this theory is correct the gene must be regarded either as a DNA-protein complex or, alternatively as a protein which requires the presence of a specific species of DNA for its action. At the moment, however, there is no real evidence to suggest that any of the nuclear proteins so far studied (see Section 1.5) is concerned with the functioning at the gene. At the same time the demonstration by Binkley (1952) that an apparently pure and protein-free preparation of RNA is capable of hydrolysing the dipeptide cysteylglycine may be taken as an indication that the nucleic acids may well possess as yet undiscovered biological properties.

The only experimental evidence which appears to cast some doubt on the concept of the gene as a macromolecule of DNA has been derived from isotope experiments on the metabolism of the nucleic acids. Whatever the material of the gene may be it must presumably be characterized by a quite exceptional degree of metabolic stability. Indeed, one might not unreasonably conceive of such material as being synthesized only when genes are duplicated and as being broken down only at the death of the cell. The early isotope experiments (see Section 1.9) did indeed lend some support to this concept of DNA as being, in tissues in which mitoses are infrequent, metabolically quite inert. More

recent experiments on similar lines have, however, given very different results. In our present ignorance of the reactions by which DNA is synthesized and broken down the interpretation of these presents some difficulties. Nevertheless it seems quite clear that, even in tissues in which mitoses are rare, there is a continuous and appreciable breakdown and synthesis of DNA (Elwyn & Sprinson, 1950; Le Page & Heidelberger, 1951; Heidelberger & Le Page, 1951; Volkin & Carter, 1951; Totter, Volkin & Carter, 1951).

Finally, if each gene is, in fact, a macromolecule of DNA, it should be possible to obtain an estimate of its molecular weight from the number of genes per set of chromosomes and DNA content per set of chromosomes. Unfortunately most of the attempts to calculate the number of genes per set of chromosomes have been carried out on Drosophila and for this species no reliable estimate of the DNA content per set of chromosomes is yet available. It has, however, been estimated that in man the number of genes per set of chromosomes is of the order of 30,000 - 120,000 (Stern, 1950; Sinnot, Dunn & Dobzhansky, 1952). We might, therefore, take the figure of 100,000 as a very rough estimate of the number of genes per set of chromosomes in the rat. In the present series of experiments it has been shown that in the same species a diploid nucleus contains rather less than 7 pg. of DNA (Table 20). A single set of chromosomes

therefore contains $^{7}/_{2}$ = 3.5 pg. of DNA. Hence the average DNA content of a single gene = $^{3.5}/_{100,000}$ = 3.5×10^{-5} pg. = 3.5×10^{-17} g. Taking Avogadro's Number to be approximately 6×10^{23}, the molecular weight of a single gene, assuming it to be a single molecule of DNA = $3.5 \times 10^{-17} \times 6 \times 10^{23}$ = 21,000,000 approximately. This figure may be compared with the estimates ranging from 35,000 to over 3,700,000 obtained for the molecular weight of isolated DNA by various workers (Table 2), and with the calculation, based on measurements of the volume of Drosophila chromosomes, that the molecular weight of the gene probably does not exceed 25,000,000 (Lea, 1946; Srb & Owen, 1952). On the other hand, calculations based on measurements of the mutagenic effects of X-rays have generally indicated that the diameter of the gene is not greater than 10 mμ. and that its molecular weight is probably of the order of 100,000 (Lea, 1946; Catcheside, 1948; Srb & Owen, 1952).

In the meantime, therefore, it would seem wise to reserve judgment on the question of the chemical nature of the gene until more evidence is forthcoming. Caution is especially necessary in view of our present ignorance of the nature and properties of the other chemical components of the nucleus. Our knowledge of the composition and structure of the nuclear proteins, for example, is still far too limited

to offer any indication of their possible function. Moreover, when it is recalled that although the nucleic acids were discovered in 1868, no real understanding of the function of DNA was achieved until Avery investigated the pneumococcal transforming factor nearly eighty years later, it will be appreciated that it would be very rash to assume, let us say, that no connection exists between the genes and the nuclear proteins merely because no such connection has yet been demonstrated. It may well be that when the nuclear protein fractions have been thoroughly investigated they will be found to contain proteins with biological properties quite as remarkable as those of the bacterial transforming factors.

Nevertheless, the fact remains that none of the other chemical components of the nucleus possesses any of the characteristics which would be expected in the material of the gene. Nuclear RNA, for example, varies in composition from one tissue to another within the same species (see Section 1.7 and Table 6), and has moreover a much greater metabolic activity than DNA (see Section 1.9). The composition of the basic proteins of the nucleus also varies from one tissue to another within the same species (see Section 1.5 and Table 9). "Residual protein", the non-histone protein found in isolated chromatin threads (Section 1.5), might be regarded as a possible material

for the gene, but the ratio "residual protein"/DNA (which, since the DNA content per set of chromosomes is constant, is proportional to the "residual protein" content per set of chromosomes) varies so much from one tissue to another within the same species (see Table 12) that this possibility also seems to be excluded. Finally, the work of Stern et al. (1952) (see Section 1.6) has shown that nuclei from different tissues of the same species have different enzymic properties. There is no reason, therefore, for assuming that there is any specially close relationship between the genes and the nuclear enzymes. The one substance which appears to remain constant, not only in amount per set of chromosomes but also in composition for different tissues of the same species, is DNA (see Section 1.2). On the basis of the evidence at present available, therefore, it seems not unreasonable to conclude that, if the gene consists of a single substance, that substance is most probably DNA. If, on the other hand, the gene is a complex of several substances, it would seem equally probable that DNA should play a major and specific part in the formation of such a complex.

Part V.

Summary of Experimental Results.

Summary of Experimental Results.

5.1 Experiments described in Part II.

1. The average deoxyribonucleic acid phosphorus (DNAP) content per nucleus has been determined in various tissues of the rat by gross chemical analysis of isolated nuclei. For the liver the DNAP, ribonucleic acid phosphorus (RNAP), lipid phosphorus (LP) and protein nitrogen (PN) contents of the whole organ were also estimated and the effects of such factors as sex, age, strain and diet were investigated. The average cell composition in the liver was calculated from the average DNAP content of its nuclei and the results of the whole tissue analysis.

2. In spleen, kidney, pancreas, small intestine, leucocytes, bone marrow, thymus, heart, lung and salivary gland the average DNAP content per nucleus is of the order of 0.65 to 0.70 pg. in both young and adult animals.

3. In the liver the average DNAP content per nucleus is of the order of 0.90 pg. in adult rats and 0.75 to 0.80 pg. in embryos and young animals.

4. In the adult rat the average DNAP content per nucleus in the liver does not vary with sex, strain or body weight and is not affected by fasting, by a protein-free diet, by a thiamine-deficient diet, by a high fat diet, by thioacetamide, by a diabetogenic dose of alloxan or by pregnancy.

5. The average DNAP content per nucleus is significantly decreased in the livers of adult rats following prolonged feeding of p-dimethylaminoazobenzene.

6. During the first four days following partial hepatectomy the average DNAP content per nucleus in the remaining fragment of the liver is 10 to 50% above the normal value.

7. The average cell mass and the average content per cell of RNAP, LP and PN in the liver are 20 to 30% lower in the normal female adult rat than in the male. In pregnancy the average cell mass and content per cell of RNAP, LP and PN are increased.

8. Fasting, protein deficiency and thiamine deficiency do not affect the number of cells in the liver but cause a decrease in the average mass and the RNAP, LP and PN content of its cells.

9. A diabetogenic dose of alloxan causes a decrease in liver weight and in the total content of RNAP, LP and PN in the liver.

10. Thioacetamide appears to have no effect on the composition of whole liver tissue although it causes an increase in the size of the nuclei.

11. Prolonged administration of p-dimethylaminoazobenzene results in an increase in the number of cells in the liver and a reduction of the average cell mass and average

content of RNAP, LP and PN. These effects occur both in livers with obvious tumours and in livers that have not reached this stage.

12. The advantages of expressing results of tissue analyses in relation to DNA content are discussed.

5.2 Experiments described in Part III.

1. The relative deoxyribonucleic acid (DNA) contents of individual nuclei in various rat tissues have been estimated by a cytophotometric method.

2. The nuclei of kidney, small intestine, pancreas and liver appear to fall into four classes with respect to their content of DNA. Class I nuclei contain approximately twice as much, Class II nuclei approximately four times as much, Class III nuclei approximately eight times as much, and Class IV nuclei approximately sixteen times as much DNA as rat spermatozoa.

3. All the nuclei of kidney and intestine appear to belong to Class I with exception of a small percentage which may belong to Class II.

4. In pancreas about 20% of the nuclei belong to Class II and the remainder to Class I.

5. In normal liver about 1 - 2% of the nuclei belong to Class III, about 40% to Class II, and the remainder to Class I.

6. In a liver infiltrated with tumour nodules induced by prolonged administration of p-dimethylaminoazobenzene it was found that about 1% of the nuclei belonged to Class IV, about 4 - 5% to Class III, about 11% to Class II, and the remainder to Class I.

7. It has been found that 48 hours after partial hepatectomy about 1% of the nuclei in the remaining fragment of liver belong to Class IV, about 15% to Class III, about 25% to Class II, and the remainder to Class I.

8. Evidence is produced to show that the coefficient of variation between the DNA contents of individual nuclei of the same Class does not exceed 5 - 15%.

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