EVIDENCE OF DIFFERENCES IN THE DESOXYRIBONUCLEOPROTEIN COMPLEX OF RAPIDLY PROLIFERATING AND NON-DIVIDING CELLS*

By DAVID P. BLOCH,† Ph.D., AND GABRIEL C. GODMAN, M.D.

(From the Histochemistry Research Laboratory, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, May 9, 1955)

INTRODUCTION

A number of investigations of the chromosomal components, desoxyribonucleic acid (DNA) and histone, suggest that both substances occur in relatively constant amounts in non-proliferating cells of diploid tissues (10, 30, 44, 12, 4, 8, 2). While in the case of DNA this constancy per genome appears to hold for all such cells within a species, differences in amounts and composition of histone among various tissues of the same organism (43, 4, 13) suggest that a histone constancy may hold only for cells of the same type. Such observations are in accord with the hypothesis that DNA is the physiologically active member of the gene complex, and that histone acts as a modifier regulating genetic expression in the various differentiated cells of an organism.

The cytophotometric technique of Pollister (41, 38) which makes possible the comparison of amounts of stained substances within individual cells of a tissue, is well suited to the demonstration of changes in the amounts of the chromosomal constituents during the different stages (interphase and mitotic) of the cell cycle. Such analyses of DNA, using Feulgen and methyl green staining and ultraviolet absorption, and of histone, by employing fast green under alkaline conditions, have demonstrated a doubling in amounts of both substances during an interphase period prior to cell division (44, 47, 19, 34, 17, 8, 2). Furthermore the increase in these substances occurs simultaneously during this period (8, 2). Relatively little is known, however, of possible qualitative changes in the chromosomal components and their interassociations during the different stages of the cell cycle. Yet these may be of prime importance in the regulation or expression of changing physiological function.

Often the conjoint application of several analytical procedures on the same

* Supported in part by grants from the Jane Coffin Childs Fund for Medical Research, The Damon Runyon Memorial Fund for Cancer Research, and an Institutional Grant of the American Cancer Society, Inc., to Columbia University.

† This work was carried out under tenure of a Fellowship from the American Cancer Society, Inc., as recommended by the Committee on Growth.

or similar preparations, can provide information concerning the physical states or associations of the nuclear substances. For example, by combining measurements both of the amounts of ultraviolet-absorbing material in living and fixed cells, and of Feulgen-staining material, Walker and Yates were able to demonstrate an ultraviolet-absorbing, non-Feulgen-staining constituent which is present in the nuclei of cells of a proliferating tissue but absent from cells which have ceased dividing (47). This substance was found to increase in amount during the period of DNA duplication and was considered by those authors as a possible low molecular weight DNA precursor, although they did not rule out the possibility of it being RNA. Swift has described differences in the basophilia of the nuclei of different embryonic tissues by staining with azure B and methyl green, although these cells were shown by Feulgen staining to have the same amounts of DNA (45).

Certain basic dyes, among them methyl green, have attributes which make them appear especially promising for the study of the relationships between various chromosomal constituents during the cell cycle. Methyl green is bound quantitatively by purified highly polymerized DNA *in vitro* (23). The use of this stain for quantitative analysis in fixed microscopic preparations is complicated by the fact that such binding would be subject to at least two variables: one being the degree of polymerization of the substrate (25, 22, 23, 46, 18), and the other, the presence of proteins which can interfere with staining presumably by competing with the dye for the anionic groups of the DNA molecule (23, 1). This suggests that the measurement of staining of the same individual cells with both the methyl green and Feulgen techniques might serve to indicate changes in the desoxyribonucleoprotein complex with regard to the degree of polymerization of the DNA moiety (22, 24), and the nature of the DNA-protein bond during the cell cycle.

Preliminary experiments in this laboratory have shown that during premitotic synthesis of chromosomal material there appears to be a close correspondence between the capacity of the nuclei to stain with both the methyl green and Feulgen techniques, indicating amounts of DNA ranging from the diploid (2 C) to twice that value (4 C). However, in a number of cells containing the 2 C amount of Feulgen-staining material there was seen to be an anomalous decrease in methyl green binding (9). Similar behavior had previously been encountered in the consecutive staining of cells with the Feulgen technique and then with the alkaline fast green technique for histone. In a recent paper the authors noted that although the amounts of histone and DNA per cell parallel one another during premitotic synthesis, occasional cells containing the 2 C amounts of DNA were found to show a marked decrease in fast green staining of the histone (8). It was subsequently found that varying the method of fixation permitted detection of many more such "aberrant" cells.

The similarity in the behavior toward the two types of stains exhibited by

this particular group of cells strongly suggested that the two phenomena might be related. The fact that the anomalous decrease in staining occurred in cells which contained only the 2 C amount of DNA further suggested that the altered staining characteristics might reflect differences between proliferating cells and those which, temporarily at least, had ceased to divide. The experiments to be described were designed to determine the differences in stainability between rapidly dividing and non-dividing cells, the cause of these staining differences, and the relationships between staining behavior and the stages of the cell cycle.

Materials and Methods

The material used was, for the most part, rat liver prepared by squashing pieces of about 1 mm.³ between two glass slides. The preparations were air-dried, then fixed in absolute methanol and stored in 70 per cent ethanol until stained. When the effects of formaldehyde on staining were to be studied, methanol fixation was followed by further fixation in 10 per cent neutral buffered formalin for various periods of time, and then washing for several hours in running tap water, followed by 70 per cent ethanol. Acetylation was carried out in pure acetic anhydride for 2 hours at 55–60°C., pepsin hydrolysis in various concentrations of crystalline pepsin (Armour) at room temperature at a pH of 1.6 for 45 minutes. Preparations which had been subjected to the action of pepsin, acetic anhydride, or formalin were treated with 0.05 per cent crystalline protease-free ribonuclease (Worthington Biochemicals Corp., Freehold, New Jersey), in double distilled water, for ½ hour at 37°C., prior to staining with methyl green. If RNA is not so removed this basic stain loses its specificity for DNA, owing to the presence of unmasked acid groups of the RNA. The RNase treatment had no apparent effect other than the removal of RNA, as shown by the similarity of the Feulgen and fast green staining of the enzyme-treated and untreated preparations.

Histone extraction was carried out in the methanol-fixed preparations by immersing the slides in 0.001 N HCl at about 4°C. and gradually lowering the pH from 3 to zero over a period of about 6 hours; and also by extraction with a molar salt solution at a pH of 3.8 (14). For extraction of extrachromosomal protein, nuclei were isolated in cold physiological saline or 30 per cent sucrose using a manual Potter-Elvehjem homogenizer. These nuclei were collected by centrifugation at slow speeds, then extracted in citric acid solutions prescribed by Daly *et al.* (14). The nuclei were then smeared on slides, air-dried, methanol-fixed, and thenceforth treated in the same manner as were the squashed preparations. After mounting the stained preparations, the nuclei were pressed between the slide and coverslip to insure a flattened preparation for the cytophotometric determinations.

Staining of the fixed preparations was carried out as prescribed by Pollister and Leuchtenberger for methyl green staining of DNA (40), by Bloch and Godman for Feulgen staining of DNA when such staining was to be followed by histone staining (8), and by Alfert and Geschwind for fast green staining of histone (4). The latter is a new technique for the selective staining of histone based upon the binding at high pH of the anionic dye, fast green, to these basic proteins. The conditions for Feulgen hydrolysis were as follows: 1 N trichloroacetic acid (TCA) at 60°C. for 25 minutes for the formalin-fixed preparations; and for 15 minutes for the methanol-fixed material. These hydrolysis times were found to give optimum Feulgen staining. The TCA was used to insure retention of histone under the acid conditions of the Feulgen procedure (8).

Amounts of DNA and histone in the stained nuclei were determined according to the cytophotometric method of Pollister and Ris (41) as modified by Moses (32). Since the nuclei of these preparations were in the shape of flattened discs, the amounts of stain were calculated by

multiplying the extinctions by the nuclear areas. The latter was taken as the product of the long and short diameters. Feulgen stain was determined at a wave length of 575 millimicrons, methyl green and fast green at 630 millimicrons. Amounts of stained material and nuclear areas are both expressed in arbitrary units. Where determinations of two or more stains were to be made on the same nuclei, the cells to be measured were mapped with a camera lucida after the initial staining to enable relocation and reidentification of these cells for subsequent measurements. All three methods of staining can be used consecutively on the same cells. If stained in the following order, methyl green, Feulgen, fast green, the prior stain may be removed without interfering with subsequent determinations (8, 9).

RESULTS

1. *The Staining Characteristics of the Cells of Proliferating Tissues.*—When amounts of Feulgen stain are plotted against methyl green stain of the individual nuclei in methanol-fixed squashed liver preparations of a 3 day old rat, the cells are found to fall into two groups (Text-fig. 1). Among the cells of group A, there exists an excellent correlation between the Feulgen and methyl green staining as the Feulgen values range from the diploid (2 C) to twice that amount (4 C). These cells include the stages during which new DNA is synthesized in preparation for division (44, 19). Group B consists of cells containing the diploid amount of Feulgen-staining DNA, but having a decreased capacity for methyl green binding. The frequency distribution of the ratios of Feulgen to methyl green shown in Text-fig. 1 (bottom) also demonstrates the two groups, the lower values belong to the cells of group A, and the higher, to those of group B.

If Feulgen-stained DNA is similarly plotted against fast green-stained histone in methanol-fixed material,¹ the same type of staining behavior is noted (Text-fig. 2). Among the cells of group A there is a parallel increase in both DNA and histone from the 2 C to the 4 C amounts as these cells prepare to enter into mitosis. The group B cells show the 2 C amount of DNA, but a marked decrease in the fast green staining of histone. As in the previous case, the two groups are also indicated in the frequency distribution diagram of the ratio of Feulgen to fast green staining.

By consecutive staining of the identical preparation first with methyl green and then with fast green, it can be seen that the same cells show the decreased capacity to bind both dyes. It should be emphasized here that the cationic methyl green and the anionic fast green stain two distinct components of the chromosome, DNA binding the former, and histone the latter. The lowered methyl green and fast green staining thus appear to be separate manifestations of related events. Because both types of stain decrease together in these cells, the frequency distributions of the ratios of staining in the two groups of cells

¹ Although formalin fixation was prescribed by Alfert and Geschwind for the staining of histone, methanol was found in these experiments to be adequate for the specific staining of histone, and essential for the demonstration of differences between the two types of interphase cells.

TEXT-FIG. 1. Top. Comparison of the Feulgen-staining and methyl green-binding capacities of DNA in two types of nuclei in squash preparations of the liver of a young rat; the parallel increase in both stains in the nuclei synthesizing chromosomal material in preparation for division (group A), and the decrease in methyl green binding in a fraction of the nuclei containing the 2 C amount of Feulgen-staining DNA (group B) are demonstrated. The scales are logarithmic for reasons stated in a previous communication (8). The diagonal is the regression of methyl green on Feulgen staining in the cells of group A. This regression, which coincides with the theoretical regression of 45° , indicates a proportionality in the increase in both types of staining within the cells of this group. Bottom. Frequency distribution curve of the ratios of Feulgen to methyl green stain in these cells. The two curves represent the ratios found in the two types of nuclei; the solid lines belong to the nuclei involved in the synthesis of chromosomal material (group A), and the broken lines, to a fraction of those 2 C nuclei which show the decreased methyl green binding (group B).

TEXT-FIG. 2. Top. Comparison of the Feulgen staining of DNA and the fast green binding of histone in the two types of nuclei of a rat liver preparation similar to that used in Text-fig. 1. Among the cells involved in the duplication of chromosomal material, there is an approximately proportional increase in the DNA and the histone, as shown by the two types of stain. A fraction of the cells containing the 2 C complement of DNA show a decreased capacity for fast green binding by the histone. The broken line is the theoretical regression of fast green on Feulgen staining. Bottom. The frequency distribution curve of the ratios of Feulgen to fast green staining. The two nuclear types, represented by the two curves, correspond to those described in Text-fig. 1, bottom.

overlap to a greater extent in Text-fig. 3 than in the previous graphs. It appears likely however that the decreases in the amounts of these two stains in the group B cells are not proportional. The difference between the ratios in the two groups is statistically significant.

Correlation of nuclear size with the various staining capacities aids in estab-

lishing the pattern of staining change during the interphase period. The typical plot of the amount of Feulgen dye bound per nucleus *vs.* the nuclear volume

TEXT-FIG. 3. Top. Comparison of the fast green binding of histone and the methyl green binding of DNA in a preparation similar to those described in Text-figs. 1 and 2, showing the identity of the cells with the decreased capacity for binding both types of dye. Bottom. The frequency distribution curve of the ratios of fast green to methyl green.

suggests that after division there is an increase in nuclear volume which is followed by an increase in DNA (Text-fig. 4).

Similar treatment of the methyl green (Text-fig. 5), and the fast green data (Text-fig. 6) show that two processes are taking place. After division, the increase in nuclear size is accompanied either by a decrease in the methyl green and fast green staining (group B), or by an increase in both (group A).

Summarizing the results described so far; it has been shown that the cells of the young rat liver can be separated into two groups. During interphase the cells of group A increase in size and at the same time increase proportionally in DNA and histone content from the 2 C to the 4 C amounts, as shown by Feulgen and methyl green staining of DNA and fast green staining of histone. The group B cells increase in size, maintaining the 2 C amount of DNA as shown by

TEXT-FIG. 4. Relationship between DNA content as indicated by Feulgen staining, and nuclear size during the interphase period of the cell cycle, in a squash preparation of young rat liver. After mitosis the nuclei increase in size, and prior to a subsequent division, double in DNA content.

Feulgen staining, but show a decreased capacity to bind both methyl green and fast green.

2. *The Cause of the Anomalous Staining Behavior of the Group B Cells.*—Depolymerization, or some similar change in the physical state of DNA, or inhibition by competing protein are the likely alternative explanations for the reduced methyl green affinity of DNA. The loss in the methyl green-staining capacity of the group B cells can only be interpreted as due to the effects of protein which competes with the dye for the binding sites of the DNA molecule. Various treatments which result in the loss of protein from the cells, such as acid extraction of histone or pepsin hydrolysis; or in the blocking of protein basic groups by acetylation or formaldehyde fixation (33), also result in an

increase in the methyl green binding. Text-fig. 7 illustrates the effect of histone extraction and acetylation of the protein basic groups on the methyl green staining of these nuclei. These were methanol-fixed squash preparations similar to those used in the previous experiments. The upper distribution curve (Text-fig. 7 A) is that of the amounts of methyl green stain per nucleus prior to either treatment. The shaded portions designate the areas containing the group B

TEXT-FIG. 5. Relationship between methyl green binding by DNA and nuclear size during the interphase period of the cell cycle—in a methanol-fixed squash preparation of young rat liver. After mitosis there is an increase in nuclear size accompanied by a decrease in methyl green binding. Prior to mitosis the methyl green binding increases to twice that of the post-mitotic cells.

nuclei. After histone extraction (Text-fig. 7 B) there is an increase in the staining of all nuclei. However, the discrepancy between the amounts of dye bound by the nuclei of the group B cells and the post-telophase nuclei of group A is still present, indicating that while histone does contribute toward inhibition of methyl green staining in all cells, there is an additional non-histone component present in the group B cells which also acts as a staining inhibitor. On acetylation (Text-fig. 7 C), there is a similar increase in the methyl green staining of the nuclei of group A, but a disproportionate increase in those of group B so that the differences between the staining of the group B and the post-telophase group A nuclei disappear. This also lends support to the idea of an additional

protein inhibitor of methyl green staining in the group B nuclei. The frequency distribution of amounts of methyl green bound in cells so treated is very similar to that obtained by Feulgen staining (Text-fig. 7 D). Methyl green staining then, when preceded by acetylation and enzymatic removal of RNA, yields results comparable to those of the Feulgen technique in the quantitative estimation of DNA. The methyl green-nuclear volume plot obtained after such

TEXT-FIG. 6. Relationship between fast green binding by histone and nuclear size during the interphase period of the cell cycle—in a methanol-fixed squash preparation of young rat liver. After mitosis, there is an increase in nuclear size accompanied by a decrease in fast green binding. Prior to subsequent division, there is an increase in fast green binding to values twice the value of the postmitotic nuclei.

treatment shows the same relationships between nuclear size and DNA content as does the Feulgen-nuclear volume plot² (compare Text-fig. 8 with Text-figs. 4 and 5).

² Two interesting aspects of the effects of acetylation on subsequent staining were noted in these experiments which at the present time cannot be explained. (a) Acetylation appears to be relatively ineffective in eliminating the staining inhibition if the preparations are exposed to acid conditions prior to acetylation. (b) Although acetylation increases methyl green staining of DNA, Feulgen staining is reduced by different degrees in the two groups of cells, precluding the use of the latter technique after such treatment. Alfert reported a lack of effect of acetylation on Feulgen staining. However, the conditions under which acetylation was carried out in the present experiments were much more drastic than those used by Alfert (1).

TEXT-FIG. 7. Frequency distribution of the amounts of DNA in a young rat liver as shown by: (A) methyl green staining after methanol fixation; (B) methyl green staining after methanol fixation and acid extraction of histone; (C) methyl green staining after methanol fixation and acetylation of protein basic groups; (D) Feulgen staining. The staining was carried out simultaneously on all three methyl green-stained slides. These preparations were treated with ribonuclease after fixation. The shaded portions of the two upper curves represent the cells of group B. The 2 C values were approximated from the post-telophase cells. These curves suggest that while histone is able to inhibit the methyl green staining of all cells, the group B cells contain an additional non-histone inhibitor. After relatively complete suppression of these inhibiting effects, and after removal of RNA, the methyl green and Feulgen staining frequency distribution curves are similarly shaped.

On the basis of our present knowledge, the somatic nuclear proteins can be divided into three fractions: the histones, the "residual protein" of Mirsky (31), and the extrachromosomal nuclear proteins (39). An attempt was made to characterize the substances responsible for the differences in the methyl green staining of the 2 C cells of groups A and B by subjecting the preparations to various treatments which are known to extract one or more of these nuclear protein components. The results of such treatments are summarized in Table I.

TEXT-FIG. 8. Relationship between nuclear size and DNA content as indicated by methyl green staining after RNase extraction of RNA and acetylation. Compare with Text-figs. 4 and 5.

Most of the extrachromosomal nuclear proteins are extracted during the isolation of nuclei from tissues using aqueous media (39). According to Mirsky and Ris (31), after extraction of isolated nuclei from a number of tissues with physiological saline followed by citric acid, all the extrachromosomal proteins are removed; only the chromosomal residual protein and nucleohistones remain. The chemical analysis of nuclear preparations obtained in this manner indicate a composition similar to that of isolated chromosomes (31, 14). When such treatment of isolated nuclei is followed by extraction of the histones with molar saline at a pH of 3.8, which according to Daly *et al.* results in complete

removal of histone (14), the differences in the methyl green-binding capacity of the two types of 2 C nuclei remains. Such treatment of isolated nuclei yields preparations that resemble in their staining capacities, the rat liver squashes from which histone had been removed by acid extraction. Presumably the only protein remaining after such treatment, aside from that of the nuclear mem-

TABLE I
Effect of Various Treatments on the Difference Between the Methyl Green Staining of the Group B Nuclei and the Post-Telophase Group A Nuclei

Differences are expressed as percentage of the staining of the post-telophase nuclei, and represent measurements on an average of 25 nuclei.

Treatment	Difference
	<i>per cent</i>
Methanol fixation	38.8 38.6 33.7
Methanol fixation, acid extraction of histone	36.6
Isolation in 30 per cent sucrose, extraction of nuclear proteins with physiological saline, then citric acid, extraction of histones with molar saline at pH 3.8, RNase	34.2
Isolation in, and extraction with 30 per cent sucrose for 24 hrs., removal of histones by acid extraction, RNase	15.8
Methanol fixation, pepsin digestion, 1.5 mg. pepsin/ml., RNase	13.8
Methanol fixation, pepsin digestion, 5 mg. pepsin/ml., RNase	6.3
Methanol fixation, ½ hr. 10 per cent neutral buffered formalin, RNase	29.7
Acetylation, RNase	6.3 4.2
Feulgen staining	6.0 0.0

brane, is the chromosomal residual protein. It is of interest that prolonged extraction of the isolated nuclei with 30 per cent sucrose results in increased affinity of group B nuclei for methyl green. The reason for this is unknown.

The effects of pepsin in eliminating the inhibition of methyl green staining accords with the concept of the protein nature of the inhibitor. The inhibitor, however, appears to be relatively resistant to the action of the enzyme, a high concentration of the latter being required for the nearly complete suppression

of the inhibition under the conditions employed in this study. Digestion with a more dilute pepsin solution results in the elimination of roughly two-thirds of the staining inhibition. Histone removal by pepsin is relatively complete, however, even at this lower concentration, as was shown by the failure of these preparations to stain with alkaline fast green. This is not surprising since crystalline pepsin has been shown to effectively degrade histones (16). The enzyme causes the loss of histones from chromosomes in which they are presumably associated with the DNA (16), contrary to the earlier reports of Mazia *et al.* (27). The resistance, though incomplete, of the non-histone methyl green staining inhibitor to the action of pepsin suggests that it is "protected," perhaps by a close association with DNA, as was previously postulated by Mazia *et al.* to explain their observed resistance of histone to degradation by this enzyme. On the basis of these experiments it is thought that the main, if not the sole component of the nucleus responsible for the staining differences between the nuclei of the two groups of cells, is the chromosomal residual protein.

The decreased fast green staining of histone in the group B cells can be partially circumvented by fixation with formalin as prescribed by Alfert and Geschwind (4) (see Bloch and Godman (8)). Even under these conditions, the variation in the amounts of histone from cell to cell is still apparently greater than that of the DNA. It is possible that the formalin acts either to prevent the loss of histone by solution or diffusion, or to detach the histone from otherwise interfering proteins. In the latter case the formalin might be expected to bind the basic groups of the histone during fixation. The subsequent removal of the formaldehyde from these groups during TCA hydrolysis would make them available for binding the acid dye. (The removal of nucleic acids is a prerequisite to the fast green staining of histone under these conditions (4), so that they cannot account for the decreased histone staining of the group B nuclei.) Whether a protein fraction acts as an inhibitor of the fast green staining of histone in a manner similar to the inhibition of methyl green staining of DNA remains to be seen. Such an inhibitory effect is possible (8). Unfortunately, attempts to block potentially inhibitory protein carboxyl groups results in non-specific staining of histones, as was previously also reported by Alfert and Geschwind (4).

3. Relationships between Staining Behavior and Cell Development.—Primarily because the cells showing the anomalous staining characteristics appear almost invariably to be those which are not synthesizing chromosomal material, it is thought that they have, temporarily at least, ceased dividing. Their nuclei generally have more prominent nucleoli than those of group A. The homologous non-proliferating 2 C cells of the adult animal have the same staining characteristics as the group B cells of the younger animal, as shown by methyl green binding before and after acetylation, and fast green staining of histone (Table II). (The higher polyploid nuclei of the adult showed the expected geo-

metrically increasing integral multiples of the amounts of dye bound by the diploid cells, regardless of treatment. Therefore the amount of dye bound by a polyploid cell, corrected for its degree of polyploidy, can serve as an indication of the amount bound by the diploid cell.) The adult liver apparently contains a cell population consisting almost exclusively of group B cells. Thus it appears that the anomalous staining characteristics of this group are associated with mitotic quiescence and functional maturity. All of the preparations described in Table II were fixed in methanol, no effort being made to insure complete retention of histone staining in the group B cells, in order to enhance the differences between the cells of the two groups.

The existence of the two types of interphase cells which are distinguishable by their staining behavior appears to be a rather general phenomenon. It has

TABLE II
Staining Properties of 2 C Post-Telophase Nuclei, the Group B Nuclei of the Young Animal, and Adult Nuclei

Values are for liver, except where indicated. Adult liver values are the means of individual values divided by polyploid class values, yielding amounts per diploid cell. Each figure represents measurements of at least 15 nuclei.

Organism and stain	Group A post-telophase	Group B	Adult
Rat, methyl green	30.7 $\pm$ 3.4	18.8 $\pm$ 3.1	18.8 $\pm$ 3.3
Rat, acetylated, methyl green	42.6 $\pm$ 5.1	39.9 $\pm$ 2.7	41.9 $\pm$ 2.7
Rat, fast green	40.8 $\pm$ 3.6	28.3 $\pm$ 5.4	30.0 $\pm$ 8.2
Chick, methyl green	8.00 $\pm$.68	5.55 $\pm$.57	Erythrocyte 8.04 $\pm$.82
Chick, fast green	7.49 $\pm$.65	5.02 $\pm$ 1.96	Erythrocyte 24.8 $\pm$ 8.0

been observed in the liver of embryonic chick, in fowl lymphocytes, in tissue cultured rat fibroblasts, and in two distinct cell populations of the young rat liver, one of which was used as the basis for most of the experiments just described.

An interesting "exception" to this bimodal type of staining behavior is found in the nucleated erythrocytes of the adult fowl. Although these are non-proliferating (B type) cells, they show no methyl green inhibition, having values comparable to the 2 C post-telophase cells of the embryonic chick liver (Table II). There is also a greater amount of histone staining in the erythrocytes than in the chick liver cells. Nucleated carp erythrocytes have been found by biochemical extraction methods to contain very small amounts of residual protein (30). These observations are in accord with the hypothesis that it is the presence or absence, or greater or lesser amounts, of the residual protein fraction which is the primary factor in determining the differences in the staining behavior of the proliferating and non-dividing cells.

Because of the possibility of confusion in trying to distinguish between the cells of the two groups with such customary terms as interphase, intermitotic, resting, metabolic, non-dividing, and the like, it is proposed that the cells of groups A and B be referred to as *autosynthetic* and *heterosynthetic* interphases respectively.³ Such a nomenclature emphasizes the synthetic nature of these cells while at the same time signifying the divergent directions of synthesis: toward creation of new cells, or in performance of the functions of the physiological mature differentiated cell.

DISCUSSION

The differences in the nuclear staining characteristics described in these experiments suggest that the interphase stage of the cell cycle might be subdivided into two separate stages: an autosynthetic phase, when the premitotic duplication of chromosomal material takes place, and a heterosynthetic phase during which the cell, temporarily at least, ceases to proliferate and functions in a manner commensurate with its state of differentiation.⁴ These staining differences, furthermore, provide a means of distinguishing between these two types of cells in a tissue when cells in the heterosynthetic phase contain appreciable amounts of residual protein. Lastly, the changes in the staining behavior imply that while this protein fraction is present in the nucleus in association with DNA during the heterosynthetic phase, it is either absent, diminished, or at least relatively dissociated from DNA during the autosynthetic phase.

These hypotheses are in accord with many of the known chemical and morphological differences between physiologically mature, synthetically active cells on the one hand, and mature, synthetically inactive *or* rapidly proliferating cells on the other. The evidence discussed in the following paragraphs which would suggest the relationship described in these experiments is admittedly circumstantial, and can be summarized as follows: (a) the association of DNA with a non-histone or residual protein fraction, (b) the involvement of residual protein in the synthetic processes of the cell, (c) the cessation of many of the synthetic processes during mitosis. On the basis of such observations a detachment of at least a part of the residual protein from the DNA as the cell approaches mitosis would not be unexpected.

A number of investigations confirm the association of a non-histone or residual protein with DNA. Recently Bernstein and Mazia extracted desoxyribonucleoprotein in the form of discrete, well defined particles, as seen under the electron microscope. These particles consisted of DNA in association with both a histone and a non-histone protein component (6, 7). Mirsky and coworkers

³ The authors are indebted to Dr. Arline Deitch for suggesting these appropriate terms.

⁴ This is not to equate proliferative activity with loss in differentiation. Mitotic cells may be as differentiated from a genetic standpoint as their related non-dividing cells of the same tissue type, although many of the characteristics associated with specialized cell function are temporarily lost to view during mitosis.

had previously described the residual protein as part of the chromosome (28, 29, 31). This substance occurs, as does the histone, in association with DNA. The DNA-residual protein complex was found to be responsible for the structural continuity of the isolated chromosomes as shown by the effects of various extractive treatments on maintenance of structure (31). The residual protein is less basic than the histone (29). It remains attached to the chromosome after extraction of histone (31), and it acts as an inhibitor of the basic staining of chromosomes (31). These last three properties are shared with the methyl green inhibitor of these experiments. That this staining inhibitor is also less basic than the histones is suggested by the fact that in the cells in which it is most prevalent, there is a decrease in staining with the acid dye fast green when used under conditions for histone staining.

On the basis of the investigations of Mirsky and his coworkers, the residual protein fraction appears to perform two distinct functions: that of structure, in holding the chromosome together, and that of synthetic activity (see below). Whether these aspects are associated with two separate components of a heterogeneous fraction remains uncertain at the present time. Two protein components other than the histones can be identified in the giant salivary gland chromosomes of *Drosophila*. One of these, the protein of the interband regions (27), would seem to be metabolically inert; the other (42), associated with the actively synthesizing "puffs" (5, 35), metabolically active. Whether both substances would come under the heading of residual protein is not known. Most of the recent work on this substance has stressed its metabolic activity. The rate of incorporation of labelled amino acid nitrogen into this fraction is higher than that into DNA or histone (14, 20), approaching the rate of incorporation into the cytoplasmic proteins. That this metabolic activity may be associated in some way with the synthesis of cytoplasmic proteins is suggested by the rough correlation between the amounts of residual protein and nuclear RNA in different tissue types, seen on comparison of the data of Mirsky and Ris (30), on residual protein, with that of Mauritzen *et al.* on nuclear RNA (26) in comparable tissues. RNA has been implicated in cytoplasmic protein synthesis (Brachet, review (11)). The presence of residual protein among different tissues also parallels the amounts of total cellular protein and synthetic activity (30).

As the cell undergoes a transition from the heterosynthetic stage to the autosynthetic and mitotic stages, it ceases to perform many of its specialized functions (48, 36). If the association of DNA with an actively metabolizing fraction of the residual proteins is expressive of genetic control over the physiological processes of the cell, it is not surprising that the approach of mitosis is accompanied by the dissolution of this complex. It is especially interesting that during pycnotic degeneration in the ovarian follicles of the guinea pig, there occur changes in the ratios of Feulgen staining to methyl green and fast green staining which parallel those occurring during the transition of the cell from the heterosynthetic to the autosynthetic interphase (3). The similarity between

mitosis and pycnosis with regard to loss in synthetic activity has been pointed out by Leuchtenberger (24) and Pollister (37). Such changes may not serve as a general description of pycnosis however. Leuchtenberger reported an increase in the ratio of Feulgen to methyl green staining during pycnosis in mammalian liver and tumor cells undergoing regressive change (24). These conflicting results might be reconciled by supposing that while in the former case pycnosis is accompanied by a decrease in non-histone protein associated with DNA, a process of DNA depolymerization might be the predominating feature in the pycnosis occurring in Leuchtenberger's material, the decrease of methyl green binding by the DNA overriding any opposing tendency accompanying the loss of protein.

The cause of the decreased histone staining of the heterosynthetic cells with fast green is questionable. The simultaneous appearance of the methyl green inhibitor and decrease in histone staining cannot be construed to show a conversion of the histone to a non-histone protein, since the apparent decrease in histone is largely an artifact. This is shown by the effect of formalin fixation, as prescribed by Alfert and Geschwind, on the fast green staining of the histones (8). Conceivably, occupation of the binding sites of the DNA molecule by the methyl green inhibitor during the heterosynthetic stages may render the histone more susceptible to loss during the staining techniques. It is also possible that the staining of histone is subject to inhibition from competing proteins, in the same manner as is the staining of DNA, in which case the residual protein itself might be considered a potential source of inhibition of both types of staining. The loss in staining may arise from changes inherent in the histone molecule itself, during the heterosynthetic stages. Daly and Mirsky have recently reported the presence of a high lysine histone fraction in non-dividing cells of certain tissues, including liver, which is not so easily precipitated by TCA (15). Changes such as this might contribute to the observed staining decrease.

The results of histone analysis using biochemical extraction techniques would support the contention that histone changes in amount and composition accompany changes in the developmental status of the cell. Horn and Anderson have reported an increase in the ratio of histone to DNA during later development of the embryo (21). Cruft *et al.*, found lower amounts of histone in tumor tissue than in the normal tissue from which the tumor was derived (13). Of course, these studies and the present work are not strictly comparable since in the former, changes reflecting tissue differentiation or "dedifferentiation" cannot be dissociated from those which accompany changes in the rate of proliferation. Furthermore, there is as yet no common basis for the comparison of the cytochemical and the biochemical extraction methods for determining histone. The identity of histone as defined by both types of techniques remains to be proven.

SUMMARY

1. Quantitative cytophotometric analysis of the interphase cells of a rapidly proliferating differentiated tissue such as liver of new born rat, indicates that these cells can be separated into two groups on the basis of their staining characteristics after methanol fixation.

2. These groups are thought to correspond to two stages of interphase. The first, called "autosynthetic interphase," comprises cells which are duplicating chromosomal material in preparation for mitosis, and shows parallel increases in the methyl green and Feulgen staining of DNA and the fast green staining of histone from the diploid (2 C) to double these values (4 C).

3. The second group is designated the "heterosynthetic interphase," during which the cell ceases proliferating and functions in a manner commensurate with its state of differentiation. In this stage Feulgen staining indicates the diploid chromosomal complement, but there is a decreased capacity of the DNA to bind methyl green and of the histone to bind fast green.

4. The difference between the methyl green binding of the heterosynthetic and autosynthetic 2 C cells is due to the presence of a protein in the former which presumably inhibits staining by competing with the dye for binding sites on the DNA. The effect of this inhibition can be removed by extracting the protein, or by blocking the protein basic groups.

5. The decreased fast green staining of histone in the heterosynthetic cells can be minimized by prolonged fixation with formaldehyde. It is thought to stem either from a similar type of inhibition, or from an increased susceptibility of the histone to loss from the cell during this stage.

6. While histone inhibits methyl green staining of DNA in all cells, the differences between the staining properties of the autosynthetic and heterosynthetic interphase cells are believed to be due to another protein, whose properties appear similar to those of the chromosomal "residual protein." It is concluded that a complex of DNA and residual protein existing during the heterosynthetic interphase is dissociated during the autosynthetic interphase.

BIBLIOGRAPHY

1. Alfert, M., *Biol. Bull.*, 1952, **103**, 145.
2. Alfert, M., *Internat. Soc. Cell Biol. Symp.*, 1955, in press.
3. Alfert, M., *Biol. Bull.*, 1955, **109**, 1.
4. Alfert, M., and Geschwind, I. I., *Proc. Nat. Acad. Sc.*, 1953, **39**, 991.
5. Beerman, W., *Chromosoma*, 1952, **5**, 139.
6. Bernstein, M. H., and Mazia, D., *Biochim. et Biophysica Acta*, 1953, **10**, 600.
7. Bernstein, M. H., and Mazia, D., *Biochim. et Biophysica Acta*, 1953, **11**, 59.
8. Bloch, D. P., and Godman, G. C., *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 17.
9. Bloch, D. P., and Godman, G. C., *J. Histochem. and Cytochem.*, in press.
10. Boivin, A., Vendrely, R., and Vendrely, C., *Compt. rend. Acad. sc.*, 1948, **226**, 1061.

11. Brachet, J., in *The Nucleic Acids*, (E. Chargaff and J. N. Davidson, editors), New York, Academic Press, Inc., 1955, **2**, 475.
12. Chargaff, E., *Experientia*, 1950, **6**, 201.
13. Cruft, H. J., Mauritzen, C. M., and Stedman, E., *Nature*, 1954, **174**, 580.
14. Daly, M. M., Allfrey, V. G., and Mirsky, A. E., *J. Gen. Physiol.*, 1952, **36**, 173.
15. Daly, M. M., and Mirsky, A. E., *J. Gen. Physiol.*, 1955, **38**, 405.
16. Daly, M. M., and Mirsky, A. E. and Ris, H., *J. Gen. Physiol.*, 1951, **34**, 439.
17. Deeley, E. M., Richards, B. M., Walker, P. M. B., and Davies, H. G., *Exp. Cell Research*, 1954, **6**, 569.
18. Devreux, S., Johannson, M., and Errera, M., *Bull. Soc. chim. biol.*, 1951, **33**, 800.
19. Frazer, S. C., and Davidson, J. N., *Exp. Cell Research*, 1953, **4**, 316.
20. Hoberman, H. D., and Peralta, P. H., *Fed. Proc.*, 1952, **11**, 231.
21. Horn, E. C., and Anderson, N. G., *Anat. Rec.*, 1953, **117**, 524.
22. Kurnick, N. B., *Arch. Biochem.*, 1950, **29**, 41.
23. Kurnick, N. B., and Mirsky, A. E., *J. Gen. Physiol.*, 1950, **33**, 265.
24. Leuchtenberger, C., *Chromosoma*, 1950, **3**, 449.
25. Leuchtenberger, C., Himes, M. H., and Pollister, A. W., *Anat. Rec.*, 1949, **105**, 587.
26. Mauritzen, C. M., Roy, A. B., and Stedman, E., *Proc. Roy. Soc., London, Series B*, 1952, **140**, 18.
27. Mazia, D., Hayashi, T., and Yudowitch, K., *Cold Spring Harbor Symp. Quant. Biol.*, 1947, **12**, 122.
28. Mirsky, A. E., and Pollister, A. W., *J. Gen. Physiol.*, 1946, **30**, 117.
29. Mirsky, H., and Ris, H., *J. Gen. Physiol.*, 1947, **31**, 7.
30. Mirsky, A. E., and Ris, H., *Nature*, 1949, **163**, 666.
31. Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, 1951, **34**, 475.
32. Moses, M. J., *Exp. Cell Research*, 1952, suppl. 2, 75.
33. Olcott, H. S., and Fraenkel-Conrat, H., *Chem. Rev.*, 1947, **41**, 151.
34. Patau, K., and Swift, H., *Chromosoma*, 1953, **6**, 149.
35. Pavan, C., and Breuer, M. E., *J. Heredity*, 1952, **43**, 151.
36. Pollister, A. W., *Anat. Rec.*, 1929, **44**, 29.
37. Pollister, A. W., *Exp. Cell Research*, 1952, suppl. 2, 59.
38. Pollister, A. W., in *Radiation Biology*, Hollaender, New York, McGraw-Hill Book Company, 1955, **2**, 203.
39. Pollister, A. W., and Leuchtenberger, C., *Proc. Nat. Acad. Sc.*, 1949, **35**, 66.
40. Pollister, A. W., and Leuchtenberger, C., *Proc. Nat. Acad. Sc.*, 1949, **35**, 111.
41. Pollister, A. W., and Ris, H., *Cold Spring Harbor Symp. Quant. Biol.*, 1947, **12**, 147.
42. Rudkin, G. T., *Rec. Gen. Soc. America*, 1955, **24**, 593.
43. Stedman, E., and Stedman, E., *Nature*, 1950, **166**, 780.
44. Swift, H., *Physiol. Zool.*, 1950, **23**, 169.
45. Swift, H., *Int. Rev. Cytol.*, 1953, **2**, 1.
46. Vercauteren, R., *Enzymologia*, 1950, **14**, 134.
47. Walker, P. M. B., and Yates, H. B., *Proc. Roy. Soc. London, Series B*, 1952, **140**, 274.
48. Wallengren, H., *Z. allg. Physiol.*, 1905, **5**, 351.