A MICROPHOTOMETRIC STUDY OF THE SYNTHESES OF DESOXYRIBONUCLEIC ACID AND NUCLEAR HISTONE*

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Plates 6 and 7

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Desoxyribonucleic acid (DNA) appears to play a primary role in the maintenance of genetic continuity of cells throughout reproduction, cell growth, and differentiation. It has been demonstrated to transmit hereditary characters in microorganisms (Avery *et al.* (3), Zamenhoff *et al.* (38)), and in the higher organisms has been shown to exhibit a constancy in composition (Chargaff (8)), and amount per cell (Boivin *et al.* (6), Mirsky and Ris (22), Swift (31, 32)) which might be expected of a genetic determinant. Another of its properties is a remarkable stability, as shown by a low uptake of labelled atoms (Brues *et al.* (7), Hammarsten and Hevesy (16)). This uptake has been directly demonstrated by Howard and Pelc (18) and Taylor and McMasters (33) to occur during synthesis of new molecules of DNA.

Less is known of nuclear histone, the basic protein associated with DNA. The Vendrelys (34) and Daly *et al.* (11) have presented evidence suggesting some degree of constancy in composition, and the works of Mirsky and Ris (22), and Alfert and Geschwind (1) indicate a possible constancy in amount per cell in cells of the same tissues. On the other hand, the Stedmans have shown that histone composition does vary from tissue to tissue of the same animal, and from various fractions of the same tissue (29). This variability probably reaches an extreme in the male germ cells of the various species of fish (Kossel (21)), fowl (Daly *et al.* (12)), plants (D'Alcontres (10)), and invertebrates (Hultin and Herne (19)) in which histone has been found to be replaced by protamine. Daly *et al.* (11), Allfrey and Mirsky (2), and Hoberman and Peralta (17) have shown that glycine incorporation into histone is lower than that into the cytoplasmic proteins, yet higher than that into DNA. It would appear that, with regard to constancy of amount per cell and stability of composition, the histones are intermediate between the relatively stable DNA and the metabolic proteins of the cell.

The work of Swift (32), Walker and Yates (35), and Patau and Swift (24), has demonstrated that DNA is doubled during interphase before cell division. Further-

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more, this substance increases geometrically in amount per cell in polyploidy and polyteny (Swift (32), Bloch (4)). The fact that the amount of new DNA synthesized is equal to the amount of DNA already present suggests that directly or indirectly DNA plays a role in its own synthesis. Whatever the other functions of DNA and histone in the cell, the function of one of the substances, and possibly both in association, is self-replication.

It was the purpose of this study to investigate some possible relationships between DNA and histone during their syntheses with the thought that determination of the sequence in which these syntheses occur might provide information about their mutual dependencies.

Until recently no methods were available for the estimation of nuclear basic proteins of individual cells, comparable to the Feulgen test for DNA (14). Lately, however, Alfert and Geschwind have devised a technique for the selective staining of these proteins based upon their binding of the acid dye fast green, at a relatively high pH (1). This provides a method not only for their specific qualitative identification, but within limits, a means for their quantitative photometric determination as well.

Preliminary experiments in this laboratory indicated that tissues stained by using a Feulgen procedure, in which trichloracetic acid (TCA) is substituted for hydrochloric acid, retained their histone and could be restained by using the technique of Alfert and Geschwind after removing the Feulgen stain (Bloch and Godman (5)). Such a procedure makes it possible to estimate relative amounts of both substances in the same individual cells during various stages in their syntheses. In this way, correlation of DNA with its associated basic protein can be made directly rather than by depending upon their relationships with a common parameter such as nuclear size, or time since last division.

Materials and Methods

Liver from adult and newborn rats and tissue fibroblasts cultured from the muscle and areolar subcutaneous fascia of young animals were the cell types employed. Squashes made of the liver were dried, fixed in 10 per cent neutral formalin, washed overnight in running tap water, and stored in 70 per cent ethanol, until used. The material was stained with a Feulgen technique in which trichloracetic acid was substituted for hydrochloric acid mole for mole, in the hydrolysis, Schiff's reagent, and the sulfite washings. The Schiff's reagent was otherwise prepared and used according to Coleman (9) except for an additional filtration after adding the TCA to the basic fuchsin. Specimens were hydrolyzed for 12 minutes at 60°C. Fields containing the nuclei to be measured were first mapped with the aid of a camera lucida to assist in reidentification. All nuclei in the mapped areas were measured, thus assuring non-selection, although the fields were initially chosen for high mitotic activity and diversity of nuclear size and staining intensity. The relative amounts of DNA in the nuclei were determined by using the microphotometric technique of Pollister and Ris (27) as modified by Moses (23). The Feulgen stain was removed by the TCA hydrolysis used to remove nucleic acids, and the preparations were then carried through the prescribed steps for fast green staining (Alfert and

Geschwind) with the following modifications: The tissue cultures were left in the staining solution overnight, followed by ½ hour in a solution readjusted to a pH of 8.0–8.1. In all preparations removal of the excess stain was carried out in absolute methanol instead of distilled water. These steps, especially the last, were necessitated by the slow diffusion of materials through the plasma clot in which the tissue cultures were grown. The previously measured nuclei were then relocated and remeasured at 625 mμ for intensity of stain. With preparations having exceptionally high extinctions, a wave length of 600 mμ was used. Walker and Yates showed cultured fibroblast nuclei to approximate flattened discs (35). The nuclei of the squashed and dried liver cells were also found to be of this shape. Therefore, the relative amounts of DNA and basic protein were calculated by multiplying extinction by nuclear area.

Millon staining was carried out on nuclei isolated by homogenizing the tissue in ice cold 30 per cent sucrose (Ris and Mirsky (28)). These were extracted overnight. After the first 6 hours, HCl in sucrose was added to some of the samples to give a normality of 0.1. This was increased over a period of several hours to 1.0 and left overnight. This resulted in the extraction of nearly all the histone as shown by the absence of fast green staining. Smears of the histone-extracted and unextracted control homogenates were made on each slide. These were then dried, collodionized, fixed in 10 per cent neutral formalin, and stained using Pollister's adaptation of the Millon test for total nuclear protein (25). This means of histone extraction was found necessary before formalin fixation, since formalin rendered histone extremely resistant to extraction. After formalin fixation attempts to completely extract histone or even to decrease greatly the fast green staining were unsuccessful.

Studies were made of the staining of purified proteins by applying drops of known concentrations to filter paper, drying, and subsequently treating them as histological preparations. For accurate determinations of the optical densities of the stained spots, strips were immersed in aniline to increase their transparency, and the extinctions determined with a Beckman spectrophotometer.

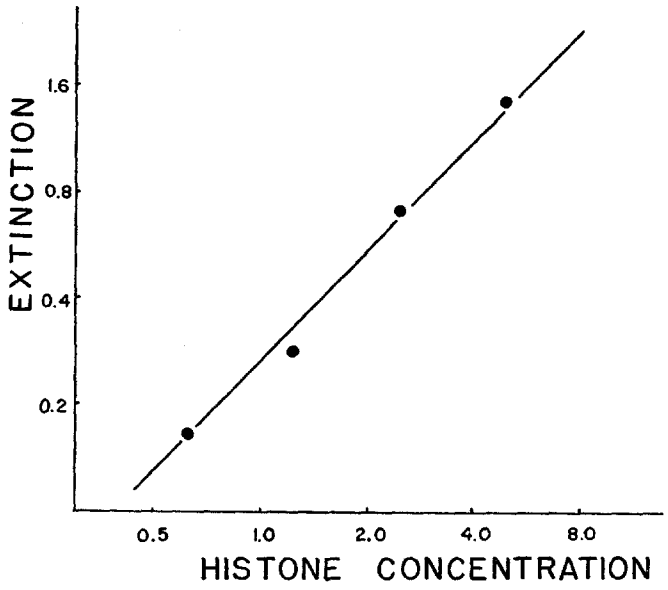
RESULTS

Validation of the Technique for Use in Microspectrophotometry.—A dye-binding method is suitable for cytophotometry when the following conditions prevail: (1) The amount of light absorbed is proportional to the number of bound dye molecules. (2) The binding is specific. (3) The number of dye molecules is proportional to the amount of binding substance present. (4) Errors in measurement due to light scattering, or distribution of the chromophore, are such that they can be assessed, or are small enough so as not to interfere significantly. It was therefore necessary to establish the validity of the fast green method for cytophotometry in regard to the above requirements.

The Beer-Lambert laws were shown to be obeyed by staining histone (Nutritional Biochemicals Corporation) which had been absorbed onto filter paper from solutions of varying concentrations. Plotting histone concentrations against extinction on a logarithmic scale (Text-fig. 1) gave a straight line with a 45° slope indicating a proportionality between dye uptake and amount of protein present over the range of concentrations used.[1]

[1] Logarithmic plots were used for several reasons. (1) The scattering of the points is proportional to the absolute values. (2) In a stepwise geometric series there is no bunching of data at one end of the plot. (3) Necessity for arbitrary selection of scales is eliminated.

However, there remains a possibility that acid substances in the fixed cell may interfere with staining by competing with the fast green for the basic groups of the protein. The capacity of certain acid substances to interfere with staining on filter paper was investigated (Fig. 1). There was no possibility of interference from DNA since this substance was removed by TCA hydrolysis. Of the other substances, pepsin depressed fast green binding the most. This was not due to proteolysis, since the two proteins were at no time present under conditions compatible with enzymatic activity of the pepsin. In all cases stain-



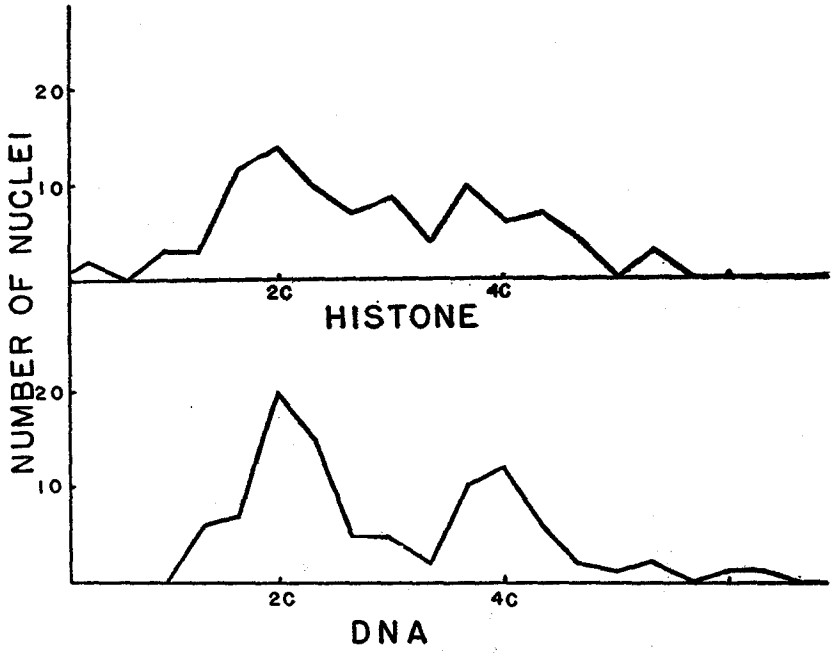
TEXT-FIG. 1. Effect of increasing concentrations of purified histone on staining with fast green. Concentrations of the droplets applied to the filter paper are in grams per 100 ml. water. Scales are logarithmic.

ing with fast green at low pH's indicated that inhibition of staining was not due to removal of proteins. Such interference of acid substances in the staining of cells remains then a possibility.

The question of specificity of the staining technique for DNA-associated protein, presumably histone, in fixed cells was sought by examination of photographs after staining with both the Feulgen and the fast green techniques (Figs. 2*a* to 2*h*). Although surrounding cytoplasm was present only nuclei stained. Furthermore, as can be seen, the distributions of the Feulgen and fast green staining materials were similar in the interphase cells as well as in the mitotic cells. This is evident on examination of such structures as heterochromatin and nucleolar associated chromatin. Two exceptions were noted to this be-

havior, both the result of cytoplasmic staining by fast green. Red blood corpuscles were sometimes seen to take a slight stain under conditions in which the cytoplasm of the leucocytes remained unstained. In the stained liver preparations, occasional cells were found to contain Feulgen-negative but fast green–positive bodies in the cytoplasm.

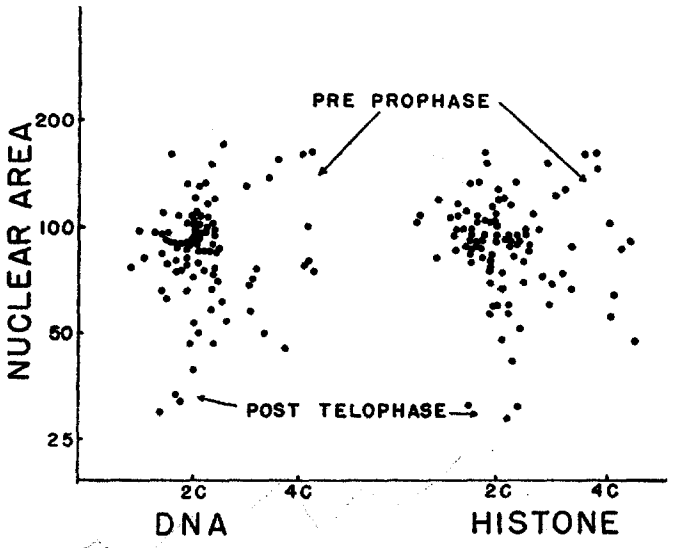
Measurements of Growing Cells.—Histograms of the distributions of the classes of nuclei with regard to DNA and basic protein (Text-fig. 2) both show the two peaks characteristic of the DNA of growing cells (Swift (31), Frazer and David-



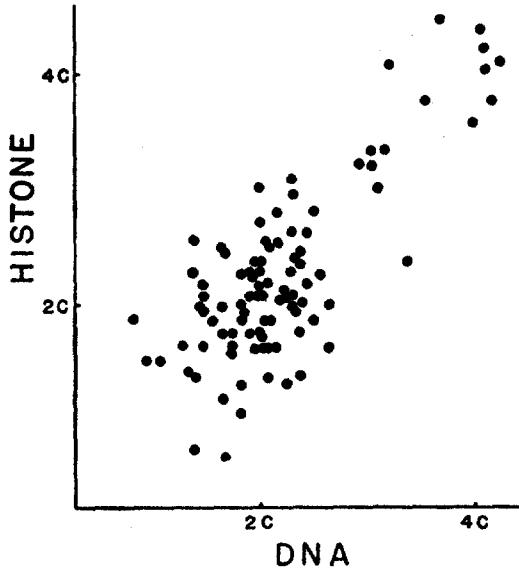
TEXT-FIG. 2. Histograms of relative amounts of DNA and histone per cell. Data pooled from several cultures of rat fibroblasts. Abscissa scale is logarithmic; ordinate, linear.

son (15)). The main peak consists of cells containing the diploid amount (2C class), and the second peak, cells which have doubled these substances in preparation for division (4C). After cell division the sequence of events, as shown by DNA (or histone) volume relationships, is an increase in size, followed by a doubling of chromosomal material (Text-fig. 3). The distributions of DNA and histone, with regard to class, and nuclear size, are similar.

Such a comparison does not prove that the syntheses of these two substances are simultaneous. This was, however, indicated by plotting directly the relative amounts of DNA against the relative amounts of histone for the identical cells (Text-figs. 4, 5). All the cells which contained the x-ploid amount of DNA also

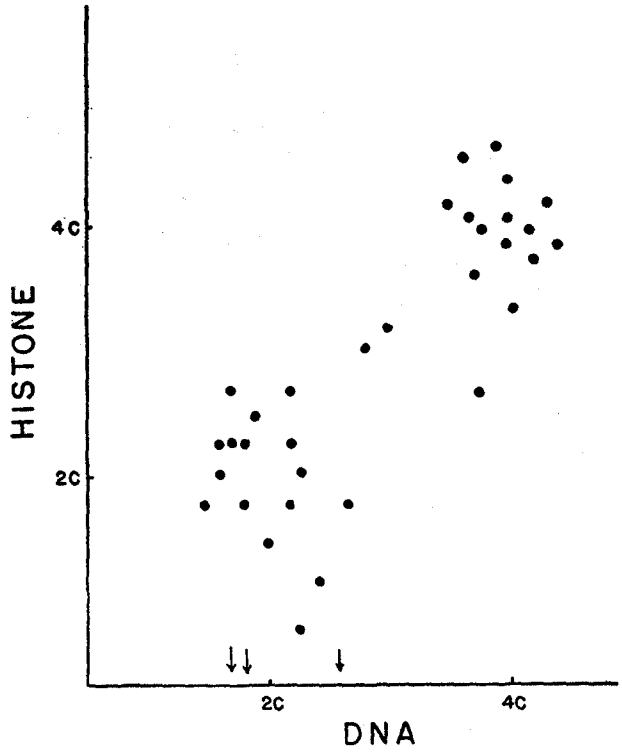


TEXT-FIG. 3. Nuclear areas plotted against relative amounts of DNA and histone. 1 day old rat liver. Logarithmic scales.



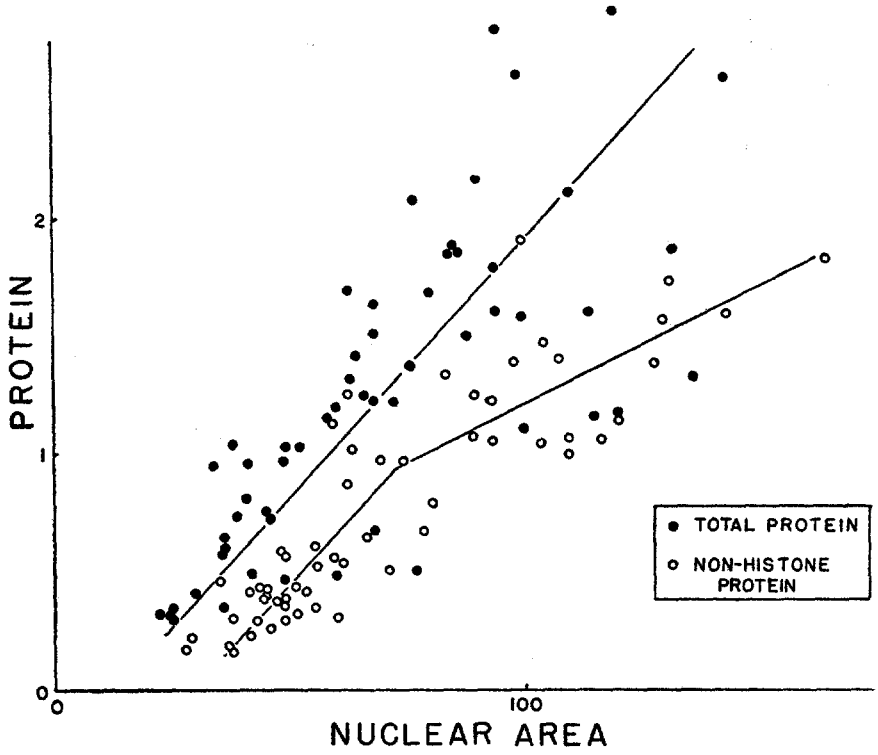
TEXT-FIG. 4. Relative amounts of DNA plotted against relative amounts of histone for the same individual nuclei of 1 day old rat liver. Logarithmic scales.

contained the x-ploid amount of histone, with the exception of three diploid cells with low histone values shown by arrows. Cells containing intermediate values for both these substances may be in the process of synthesis. The presence of these intermediates is further evidence that the syntheses of DNA and of histone are simultaneous and not consecutive.



TEXT-FIG. 5. Relative amounts of DNA plotted against relative amounts of histone in growing cultures of rat fibroblasts. Logarithmic scales.

The means of the classes of DNA and histone in growing tissues occurred in the ratio of approximately 1:2, and in the adult liver, 1:2:4 (Table I), the higher values in the latter case being due to polyploidy. The values of the different preparations could not be compared because they were stained separately. In most cases the percentage standard deviation of the histone values was greater than that of DNA. This was found especially in the diploid classes of growing tissues. The great variability of the stain obtained from one staining treatment to the next makes it essential that tissues to be compared must be carried through the staining procedure together.



TEXT-FIG. 6. Nuclear areas plotted against relative amounts of total protein, and non histone protein, of isolated young rat liver nuclei. Scales are linear.

TABLE I

Relative Amounts of DNA and Histone in Cells of Rat Liver and Rat Fibroblasts

Amounts expressed in arbitrary units. Interexperimental values cannot be compared.

Experiment	DNA			Histone		
	2C	4C	8C	2C	4C	8C
A. Adult rat	35.8 ± 4.0	75.3 ± 5.6		26.0 ± 4.4	51.8 ± 5.4	
Day old	34.6 ± 4.2			22.5 ± 4.4		
B. Adult	38.4 ± 3.9	87.3 ± 8.1	178.5 ± 23.2	15.8 ± 3.5	28.3 ± 5.5	61.2 ± 9.0
C. Day old	35.2 ± 6.1	69.2 ± 8.4		25.8 ± 4.7	52.2 ± 10.0	
D. Culture	32.3 ± 3.6	58.2 ± 3.3		15.6 ± 3.1	27.4 ± 6.1	
E. Culture	25.9 ± 2.4	54.8 ± 11.0		25.0 ± 4.9	52.4 ± 10.8	

Text-fig. 6 indicates the relationships between total nuclear protein, non-histone protein, and nuclear size, as determined by Millon staining. The non-histone fraction probably includes Mirsky's residual protein and possibly

structural proteins. Most globular proteins probably will have been removed by the extraction (Pollister and Leuchtenberger (26)). The graph here is non-logarithmic. Although the scattering of values is too great to allow any conclusions to be drawn from the Millon staining alone, the results seem to be in accord with those obtained with fast green. The lines which have been drawn are idealized to show what might be expected on the basis of the results obtained with fast green. This would be a constant difference between total and non-histone protein over the range of nuclear sizes during the time when histone is constant, followed by an increasing difference over the range in which histone is being synthesized.

One of the earlier hypotheses proposed to explain nuclear staining with fast green provided for a blocking of the basic groups of the proteins with formaldehyde, except where protected by linkage with DNA. Subsequent removal of DNA would leave exposed protein free to bind fast green. It was found, however, that exposure of histone models, and tissues after removal of DNA, to the action of formaldehyde did not prevent binding of the dye after further TCA hydrolysis. Furthermore, treatment of non-histone proteins with DNA prior to fixation did not enhance their stainability with fast green.

Therefore the staining is not simply due to the binding onto basic protein sites previously protected from formaldehyde by linkage with DNA. The correspondence between the nuclear volume histone relationships as shown by Millon's and fast green staining reinforce this point of view.

DISCUSSION

The adherence of the stain to the Beer-Lambert laws, and the similar distributions of the nuclear basic protein and the DNA, suggest that error in measurement of the stains should be similar for both substances. The substantially greater standard deviation of the basic protein values might arise through competitive interference with the staining, or through actual variations of the histone, or both. The error due to possible competitive inhibition by acid substances cannot be discounted although such error, if it does occur to different degrees in different cells, is still not great enough to mask a pattern of synthesis similar to that of DNA. Probably much of this difference in standard error reflects a greater variation of the histone either in amounts per cell, or in amino acid composition. That such variability exists has clearly been demonstrated by the Stedmans (29), the Vendrelys (34), Daly *et al.* (11), Khouvine *et al.* (20), and others.

The composition of DNA, on the other hand, appears to be unvarying from tissue to tissue of all organisms within a species (Chargaff (8)). The mean amounts of DNA per cell have also been shown to be the same throughout many types of cells within a species as shown by biochemical extraction techniques (Vendrelys (34), Boivin *et al.* (6)), and cytochemical methods (Swift (31, 32)).

In the light of such evidence it would be hard to imagine a strict histone constancy similar to that of DNA. Perhaps while the DNA content typifies that of all cells within a species of organism, the histone complement is characteristic of all cells within a cell type. Such a hypothesis would be required for several roles which have been proposed for histone in cell differentiation. The Stedmans have suggested that histones may regulate the expression of the genes of the differentiated cell by a genetic inhibitory effect (29). Danielli goes further in suggesting a mechanism of action, in which histone prevents protein synthesis by combining with ribonucleic acids (RNA). These latter would otherwise act as "trapping agents" by combining with proteins and thereby shift the equilibrium in the direction of continued protein synthesis (13). Stern, on the other hand, proposed that histones act by "locking in" a specific DNA configuration which would genetically determine the pattern of cell modulation (30). Although the apparent association of histone with DNA rather than RNA would preclude Danielli's hypothesis, as he pointed out, the higher amounts or greater basicity of histones in such highly differentiated cells as sperm and blood cells would seem to support a genetic inhibitory role. As the Stedmans have suggested, the protamines may be extreme forms of histone, playing a similar role.

In considering the various components of the gene, no less important than the manner in which these substances affect the cell, is the way in which they are duplicated, or synthesized. A second role for histone in the duplication of the gene might also be suggested. The simultaneous syntheses of DNA and histone before division lead to the interesting possibility that in the synthesis of nucleoprotein perhaps each moiety of the molecule acts by serving as a template for the synthesis of the other. This would do away with the necessity of self-duplication in a strict sense, the histone partner serving as a catalyst in the synthesis of its DNA, and *vice versa*. However, DNA might well be able to carry on the function of self-replication in a more independent manner. A mechanism has been brought within the range of imagination by the models of Watson and Crick (36), and Wilkins *et al.* (37). This model, made convincing by corroborative studies of molecular configuration through x-ray data, and application of previous knowledge of the proportions of purines and pyrimidines in DNA, consists of a two stranded spiral structure, each strand of which might synthesize the other complementary structure by acting as a template.

The close association of histone and DNA in the cell, as is readily seen by their similar distributions in fixed and stained cells, is further evidence that they may be combined in living cells. Although not conclusive, it is a step closer than was shown by the biochemical extraction of desoxyribonucleohistone. Furthermore, their simultaneous, or nearly simultaneous syntheses suggest that perhaps the two synthetic processes are also inextricably connected. Whether they can be dissociated or independently blocked remains to be seen.

SUMMARY

1. The fast green stain of Alfert and Geschwind for nuclear basic protein is shown to obey the Beer-Lambert laws when used on purified histone. Interference from acid substances other than nucleic acids as a possible source of error is indicated.

2. Use of this technique after a modified Feulgen stain enables determination of relative amounts of desoxyribonucleic acid and histone in the same individual cells.

3. DNA and histone are shown to have the same distribution in formalin-fixed nuclei.

4. The syntheses of DNA and histone proceed simultaneously resulting in the doubling of both these substances prior to cell division.

5. The standard error for histone values is greater than that for DNA; however, the source of this variability is not known.

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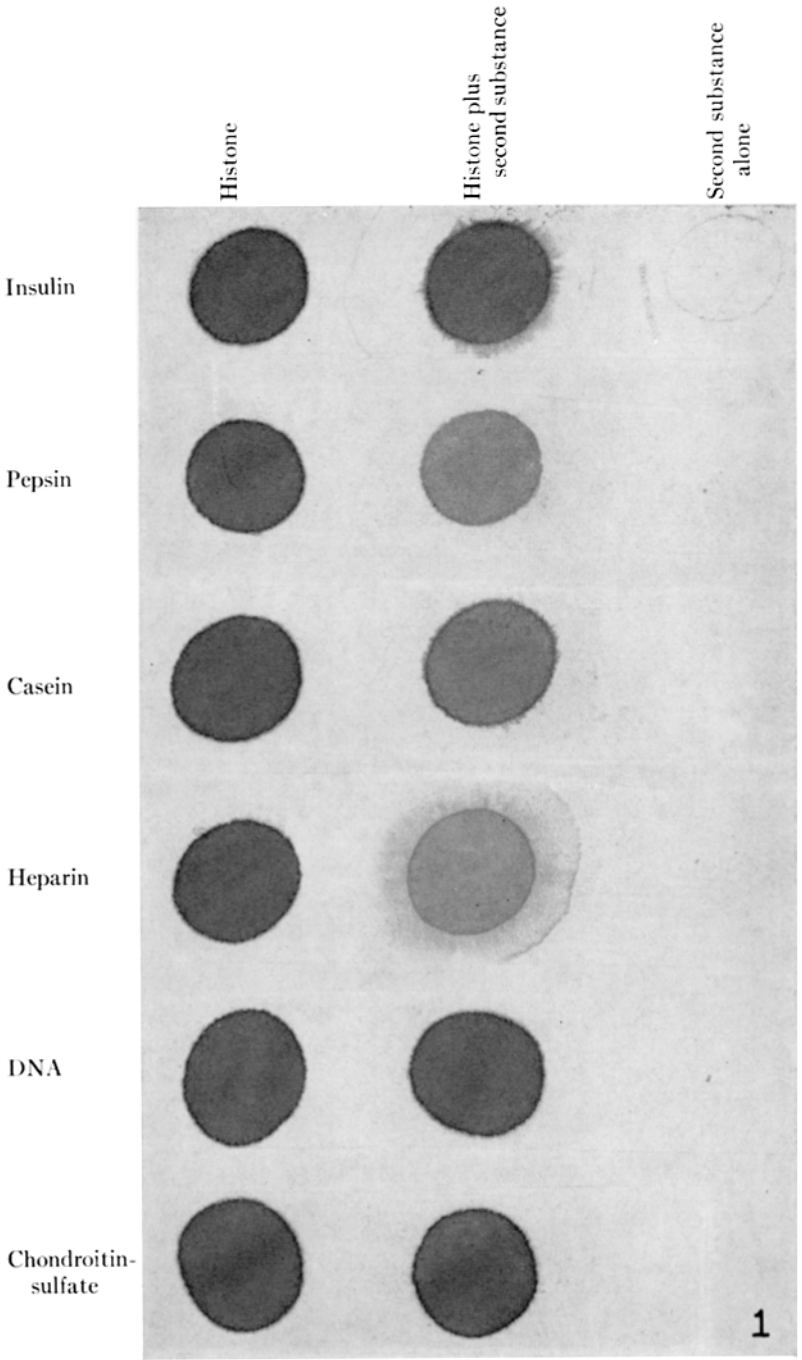
EXPLANATION OF PLATES

PLATE 6

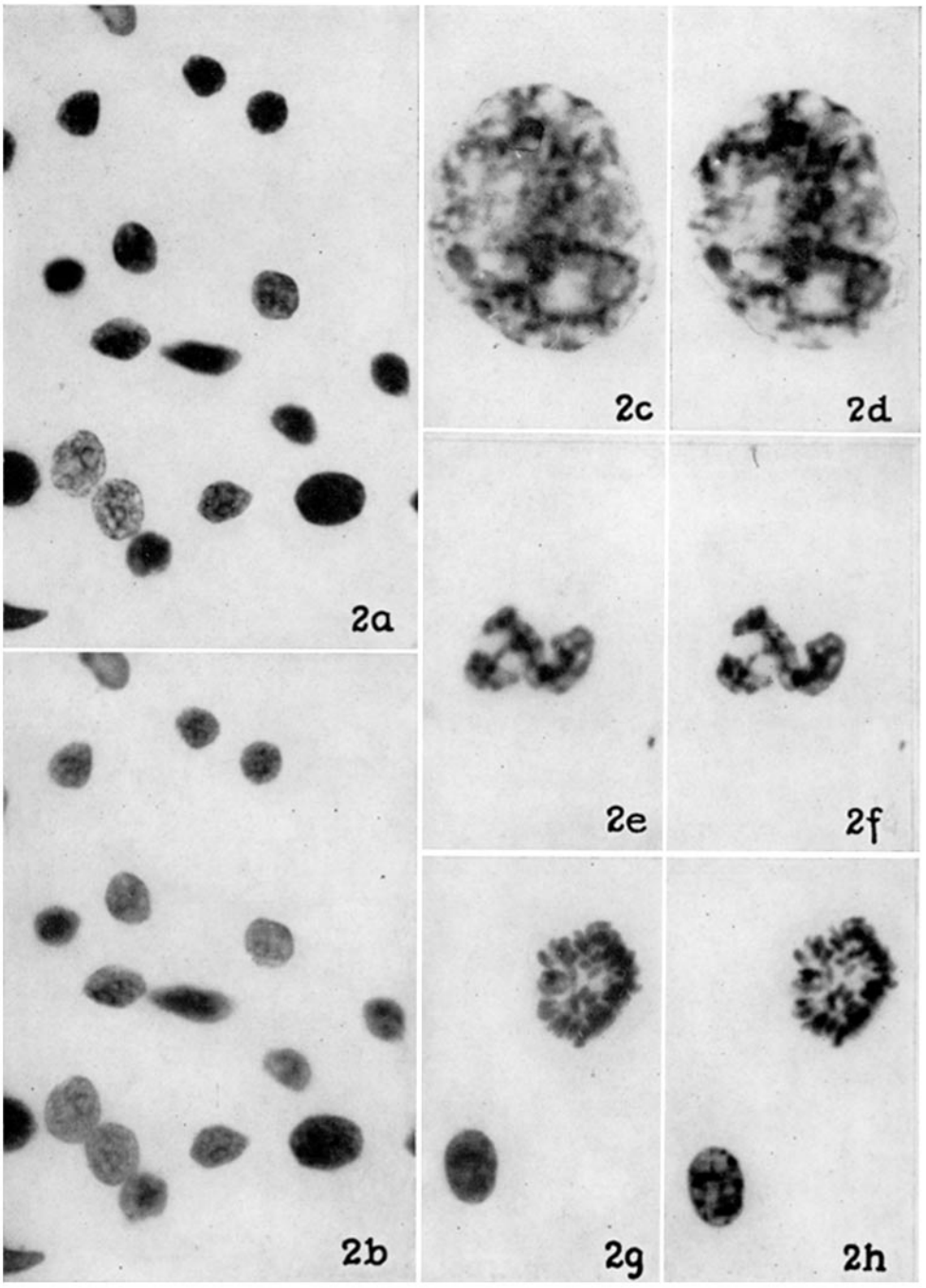
FIG. 1. Inhibitory effects of various substances on histone staining. Lack of DNA inhibition is due to the removal of this substance during TCA hydrolysis. Original concentrations of all substances were 1 per cent.



(Bloch and Godman: Desoxyribonucleic acid and nuclear histone)

PLATE 7

FIGS. 2*a* to *h*. Distribution of DNA and histone as shown by Feulgen and fast green staining. *a*, Feulgen-stained rat liver nuclei; × 800. *b*, same field, stained with fast green; × 800. *c*, and *d*, rat ascites tumor nucleus, stained with Feulgen and fast green; × 3600. *e*, and *f*, human leucocytes, stained with Feulgen and fast green; × 2000. *g*, and *h*, rat ascites tumor pro-metaphase, stained with Feulgen and fast green; × 1900. Feulgen photographs were taken with a Wratten No. 58 filter, fast green, with a No. 1240-8 red filter (Gamma Instrument Company, Inc.).



(Bloch and Godman: Desoxyribonucleic acid and nuclear histone)