# CHANGES IN NUCLEAR HISTONES DURING FERTILIZATION, AND EARLY EMBRYONIC DEVELOPMENT IN THE PULMONATE SNAIL, *Helix aspersa*

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ABSTRACT

Calf thymus histones comprising two fractions, one rich in lysine, the other having roughly equal amounts of lysine and arginine, *Loligo* testes histones rich in arginine, and salmine, are compared with respect to their amino acid compositions, and their staining properties when the proteins are fixed on filter paper. The three types of basic proteins; somatic, arginine-rich spermatid histones, and protamine can be distinguished on the following basis. Somatic and testicular histones stain with fast green or bromphenol blue under the same conditions used for specific staining of histones in tissue preparations. The former histones lose most or all of their stainability after deamination or acetylation. Staining of the arginine-rich testicular histones remains relatively unaffected by this treatment. Protamines do not stain with fast green after treatment with hot trichloracetic acid, but are stained by bromphenol blue or eosin after treatment with picric acid. These methods provide a means for the characterization of nuclear basic proteins *in situ*. Their application to the early developmental stages of *Helix aspersa* show the following: After fertilization the protamine of the sperm is lost, and is replaced by faintly basic histones which differ from adult histones in their inability to bind fast green, and from protamines, by both their inability to bind eosin, and their weakly positive reaction with bromphenol blue. These "cleavage" histones are found in the male and female pronuclei, the early polar body chromosomes, and the nuclei of the cleaving egg and morula stages. During gastrulation, the histone complement reverts to a type as yet indistinguishable from that of adult somatic cells.

## INTRODUCTION

The replacement of a "typical" histone by a protamine or an arginine-rich histone is of fairly general occurrence during spermiogenesis (1, 5, 8, 10, 12). The significance of this change is unknown. Because of its possible implication in the development of the fertilized egg, the fate of the basic sperm protein and its derivatives during fertilization and subsequent development of the egg is of particular interest.

Alfert has recently shown that in the mouse, immediately after fertilization, the sperm nucleus, or male pronucleus, loses its ability to bind the

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acid dye fast green at alkaline pH's (2). Acid dye binding under such conditions is a characteristic property of histones (3), and the loss of stainability is indicative of a change in the character of the material of which the male pronucleus is composed. This decrease of histone staining is reminiscent of a similar behavior noted in rat fibroblasts, and in liver cells during a period of interphase, which was thought to be due to masking of the histones by other substances associated with the chromosome (4), particularly the "residual protein" of Mirsky and Ris (15). Alfert attributed the loss of fast green staining of the mouse pronucleus to masking. His reasons were: (*a*) retention of the basic protein in the pronucleus, notwithstanding its loss of acid stainability, was suggested by a positive Sakaguchi test for arginine; and (*b*) loss of acid staining, noted also in interphase cells during early cleavage, did not hold for the mitotic chromosomes, which are thought to be exempt from masking by other substances (4).

We had noted a similar behavior of the male pronucleus in *Helix aspersa*. There is an important difference, however, between the mouse and the *Helix* sperm, which suggested an alternative explanation for the lack of staining in the *Helix* pronucleus. In mouse, the sperm stain strongly by the alkaline fast green method for histones up to the point of entry of the sperm into the egg (2). In *Helix*, on the other hand, the mature sperm do not stain with the alkaline fast green method (5), but behave in a manner similar to that of salmon sperm (1), which contain a protamine. Protamine is lost during this staining procedure. Lack of staining in the *Helix* pronucleus might then be more readily interpreted as indicating the presence of a protamine, derived from the sperm, rather than to a masking of histone. Indeed, loss of staining in the mouse pronucleus might suggest a transition to a protamine occurring after fertilization, rather than during sperm differentiation and maturation.

Because of the relatively small amounts of chromosomal material in the egg, extraction and chemical characterization of the chromosomal proteins from eggs would be a difficult task. The purpose of the present experiments is to describe cytochemical procedures that permit characterization of the various histones *in situ*, and the application of these methods to the histones of the fertilized egg and the developing embryo.

## MATERIALS AND METHODS

High lysine and high arginine histones were prepared from calf thymus according to the method of Daly and Mirsky (7). Histones and protamines were obtained from frozen *Loligo opalescens* using the procedure of Hamer (10). In this latter procedure the testes, the duct leading immediately from the testes, and the spermatophores from about 50 squid were pooled separately, and the histones extracted from each of these organs. Histological examination of the testes showed them to be full of sperm cells, late spermatids, and relatively few cells which appeared to be in stages earlier than spermatid. The duct and the spermatophores contained only sperm. Protamine sulfate (salmine) was obtained from Nutritional Biochemicals Corporation, Cleveland.

One per cent solutions of these proteins were prepared, and single drops containing roughly ½ milligram were applied to filter paper. These were dried, fixed for 15 minutes in undiluted neutral buffered formalin, washed briefly, and subsequently treated as histological preparations during the following staining procedures.

*Alkaline Fast Green Procedure:* This method was used as prescribed by Alfert and Geschwind (3) and consists of fixation in neutral buffered formalin (10 per cent formalin was used on histological preparations), hydrolysis at 90°C. for 15 minutes in 5 per cent trichloracetic acid, washing in three changes, 10 minutes each, of 70 per cent ethanol, staining in a 0.1 per cent solution of fast green FCF brought to a pH of 8.2 ± 1 with sodium hydroxide, and differentiation for at least 5 minutes in distilled water. Alternatively, slides were coated with collodion and hydrolyzed at 60°C. for 3 hours instead of at the higher temperature for shorter times.

*Picric Acid–Eosin Procedure:* This method is essentially similar to the alkaline fast green procedure with the following modifications: Hydrolysis is effected by a saturated solution of picric acid at 60°C. for 6 (or more) hours. The washing with 70 per cent ethanol is eliminated. A 0.1 per cent solution of eosin Y at a pH of 8.2 ± 1 is substituted for fast green. The slides are allowed to remain in eosin for at least 3 hours.

*Picric Acid–Bromphenol Blue Procedure:* The material is hydrolyzed as above with picric acid, then brought to a 0.1 per cent aqueous solution of bromphenol blue at a pH of 2.3 and allowed to stand for at least 6 hours. Differentiation was carried out as described previously (5) in slightly basic 95 per cent ethanol.

*Blocking of Lysine:* Deamination was carried out by the Van Slyke reaction (18). The samples were immersed in 2 changes, 15 minutes each, of a solution containing 5 per cent trichloracetic acid and 5 per cent sodium nitrite. The solutions were prepared

immediately before use. Alternatively, material was acetylated, usually after fixation, according to the method of Monné and Slatterback (16). The filter paper, or slides, were brought through a graded series of alcohols to absolute ethanol, then to acetic anhydride containing approximately 1 per cent glacial acetic acid. Acetylation was carried out at 60°C. for 1 hour. The material was then brought to absolute ethanol, and through a graded alcohol series to water.

*Chromatography:* The histones were hydrolyzed and the hydrolysates chromatographed according to the method used by Scherbaum *et al.* (17). 0.5 mg. of histones was hydrolyzed in 200 μl. of 3 N hydrochloric acid for 7 hours at 15 pounds pressure. The clear hydrolysates were dried, redissolved in 10 per cent isopropanol, and the total sample applied to Whatman No. 3 filter paper. The paper was developed in the first dimension in a mixture containing 4 parts normal butanol, 1 part glacial acetic acid, and 1 part water; in the second dimension, in 4 parts 90 per cent aqueous phenol, 1 part water, the solution containing 0.08 per cent hydroxyquinoline. A 0.5 per cent aqueous solution of ammonium hydroxide was placed in an open beaker during the second run. The paper was washed in petroleum ether, and the amino acids visualized with 0.5 per cent ninhydrin dissolved in acetone.

*Preparation of Snail Eggs:* Eggs taken at various times after laying, and eggs removed from the oviduct of an animal which had been laying, were fixed for several hours in 10 per cent neutral buffered formalin. During the early part of fixation eggs were brought to a temperature of 95°C. for 5 minutes to insure penetration of the fixative, were allowed to cool to room temperature, and fresh fixative added. Occasionally, the eggs were stored for several days in fresh fixative. The eggs were then opened and the ova within removed and processed in the usual manner for paraffin embedding and sectioning. (Similar heating of ovotestes during fixation did not in any way affect the staining properties.)

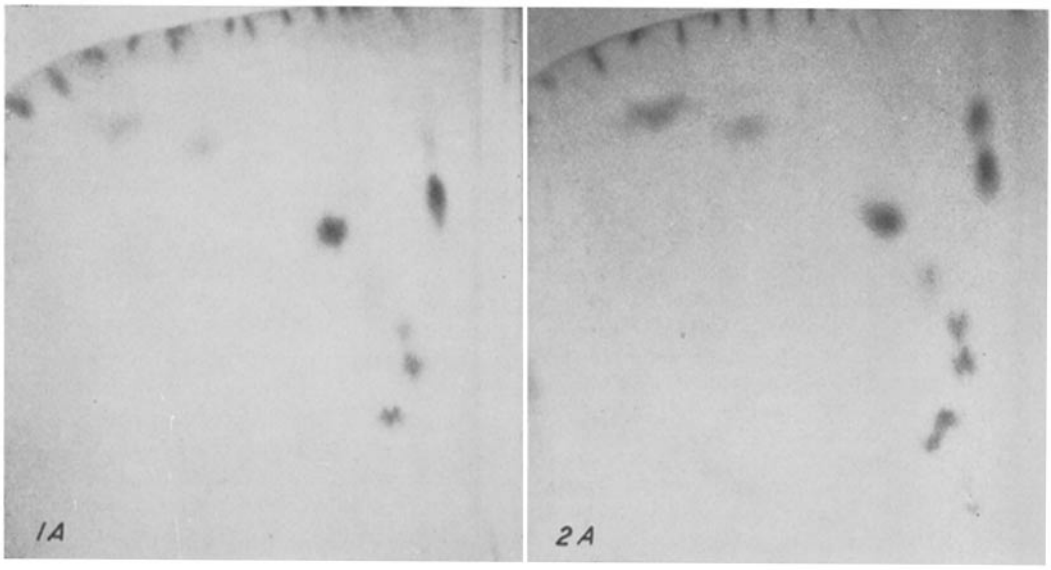
## OBSERVATIONS

### *Cytochemical Characterization of Histones*

Histones (and other proteins of high isoelectric point) are preferentially stained in the cell by acid dyes at high pH. Because the major contributors to the basicity of histones are arginine and lysine, blocking of lysine at least, and its effect on staining, can be used to determine the prevalence of this amino acid as compared with arginine, in the histone. Furthermore, the lability of protamines can be used to distinguish these substances from other histones. Figs. 1 through 4 illustrate how such procedures can be used to differentiate between somatic histones, the arginine-rich histones found in spermatids and immature sperm, and protamines.

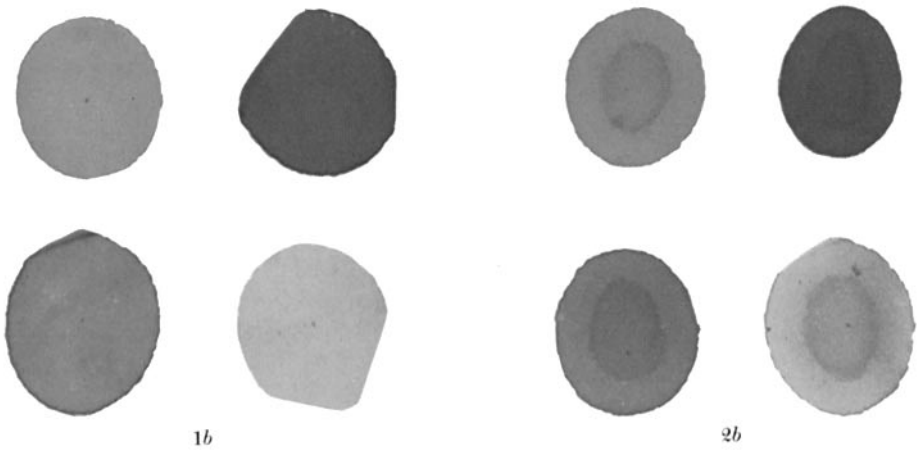
The high lysine and high arginine histones obtained from calf thymus (Figs. 1 and 2) represent two extreme forms found in somatic tissues. Both stain with the alkaline fast green, the picric acid-eosin, and the picric acid–bromphenol blue procedures. The staining of each is inhibited to some degree by blocking lysine. Inhibition is almost complete in the case of the high lysine histone. The effect of such blocking on bromphenol blue staining is shown here because of the greater reproducibility of this method when used on the filter paper spots. When applied to tissues, lysine blocking results in the loss of staining of somatic tissues by any of these methods. The greater part of somatic histones then consist of histones in which lysine predominates. The presence of small amounts of arginine-rich histones and protamines such as occur in sperm cells cannot be ruled out. Such substances, if they do occur in somatic tissue, are not localized to any great extent within one cell type. Little inhibition of staining is seen after blocking of lysine, in the arginine-rich histones obtained from immature sperm (Figs. 3 and 4). Lysine contributes relatively little to the over-all basicity of these proteins. (These histones have a slightly higher ratio of arginine to lysine than does the high arginine histone obtained from calf thymus.)

Protamines behave in a manner similar to that of the arginine-rich histones, with regard to the relative lack of effect of lysine blocking on staining. However, they differ from more typical histones by virtue of their lability during extraction of nucleic acids with trichloracetic acid or any other acid in which protamine is soluble. Their demonstration depends upon their precipitation and their retention in the form of insoluble complexes during the staining process. Picric acid, which is sufficiently acid to effect removal of DNA, forms a highly insoluble precipitate with all histones, and also protamine. Eosin Y and bromphenol blue both form similar insoluble complexes. These methods then permit retention and staining of protamines which can otherwise be differentiated from the more complex histones by their loss during the trichloracetic acid hydrolysis step of the alkaline fast green method.



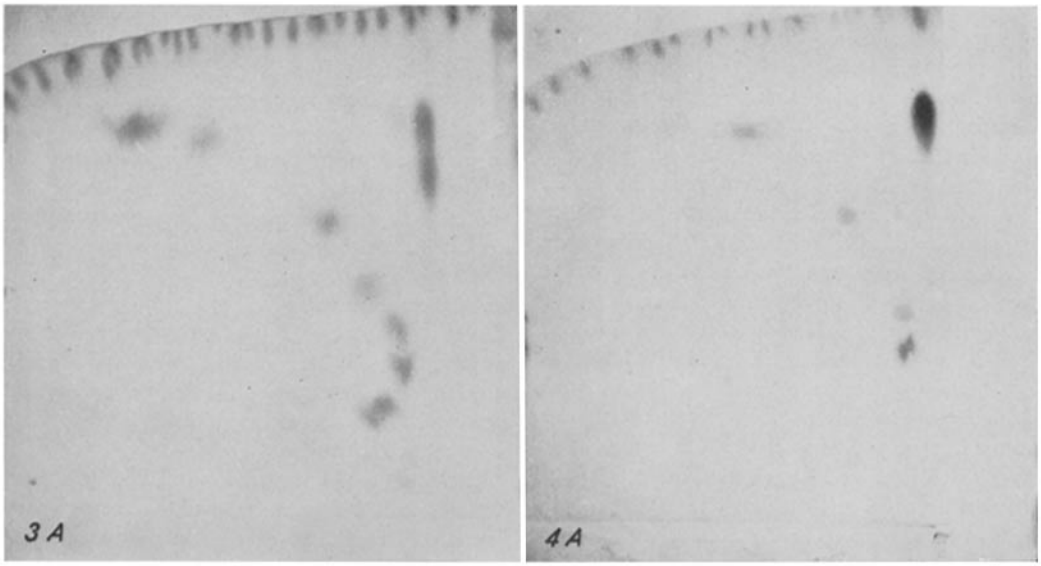

FIGURES 1*a* through 4*a*

Chromatograms of hydrolysates of calf thymus high lysine histone (1 *a*), calf thymus high arginine

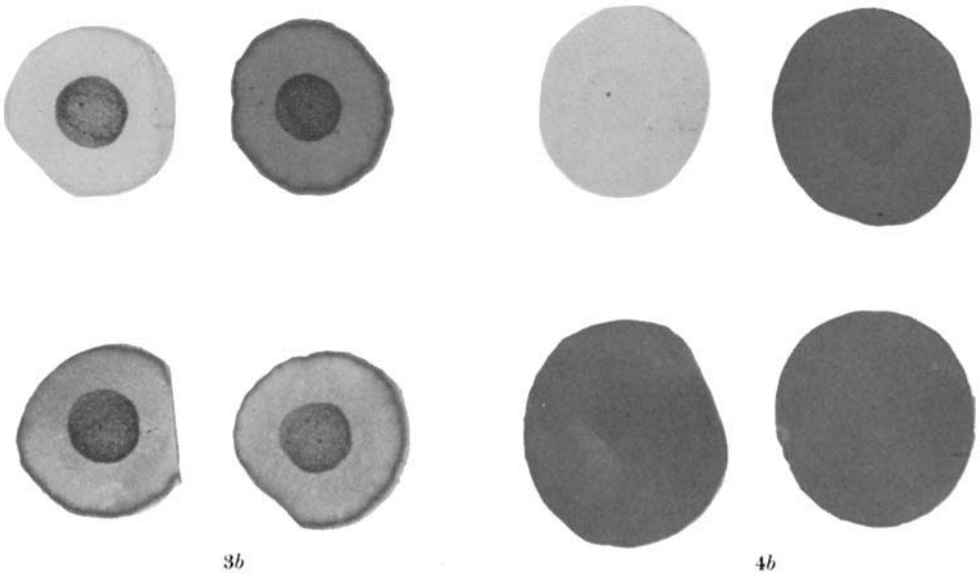



FIGURES 1*b* through 4*b*

Spots of the above histones on filter paper, stained with the fast green, the picric acid eosin, and (acetylation in the case of salmine). The chart on page 75 indicates the positions of the spots.




histone (2 *a*), *Loligo* testes histone (3 *a*), and salmine (4 *a*), respectively.




the bromphenol blue procedures, the latter, with and without prior deamination with nitrous acid

*Characterization of Histones during Spermatogenesis, Fertilization, and Cleavage*

As reported previously (5), the histones of the early spermatid and all prior spermatogenic stages in *Helix aspersa* stain with alkaline fast green, but do not so stain after deamination or acetylation. During spermiogenesis the following changes occur. In the "spade" stage, the nuclei stain slightly even after deamination. In the later elongated stage, the nuclei stain heavily after deamination. Blocking of lysine has no apparent effect on staining, although no measurements of staining were made. During sperm maturation, the basic protein becomes increasingly labile to TCA hydrolysis, and the protein of the mature spermatophore sperm no longer stains with the alkaline fast green procedure whether or not lysine is blocked. However, these sperm stain strongly with alkaline eosin or with bromphenol blue, after picric acid hydrolysis. These staining changes were interpreted as indicating a transition to an arginine-rich histone in the spermatid, and a protamine in the sperm (5).

After fertilization, the male pronucleus remains approximately the same size as the sperm head for a time (Figs. 5 and 6). Changes in staining characteristics cannot be attributed to dilution of existing nuclear material. The nucleus can no longer be detected by either the fast green or the picric acid–eosin staining procedures. A very faint staining with picric acid–bromphenol blue can be seen, which is in contrast to the relatively intense staining of the mature sperm head (Fig. 6, insert). The bromphenol blue staining here is absolutely specific for nuclear material. The remainder of the photographic image in Fig. 6 is due to refractility of the egg. The Sakaguchi test shows that the concentration of protein-bound arginine in pronuclei is no greater than that of surrounding cytoplasm, in contrast to the very strong Sakaguchi reaction exhibited by the mature sperm. The protein of the male pronucleus is basic as compared with the remainder of the ovum, as indicated by its positive though slight affinity for bromphenol blue under these conditions. It is not a protamine, or an arginine-rich histone, as evidenced by the loss of staining by bromphenol blue after acetylation or deamination. The protein, by virtue of its basicity and association with DNA, is designated as histone, and is apparently different from the histones of the more adult organism, particularly in its low basicity.

At fertilization, the ovum is in the metaphase of the first meiotic division. After fertilization the maturation divisions are completed, and the system consists of three polar bodies, and two pronuclei. The latter greatly increase in size prior to fusion. The male and female pronuclei can often be distinguished by their position in the cell and disposition of the chromatin. The female pronucleus occupies a more peripheral position than the male. Its chromatin forms a skein in an otherwise seemingly empty vesicle. The male pronucleus after enlargement is similar; however, discrete chromosomes can be seen. These are long and thread-like, at first, but later assume, with the chromosomes of the female pronucleus, a more condensed aspect typical of a late prophase or metaphase chromosome.

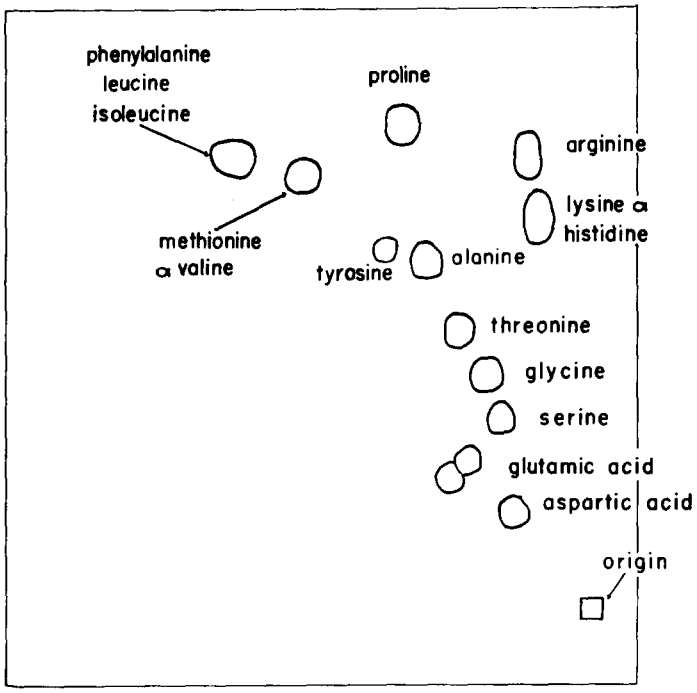
The condensed chromatin, whether in the form of the spherical chromosomes of the polar bodies, the skein or thread-like chromosomes of the pronuclei (Figs. 9, 10), the chromosomes of the maturation divisions of the ovum (Figs. 7, 8), or of the first cleavage division of the fertilized egg, all exhibit the faint but definite staining with bromphenol blue as described for the early male pronucleus. None of the alternative histone procedures yield a positive test. Bromphenol blue staining is lost on acetylation or deamination. Non-specific staining is occasionally encountered in the cytoplasm of one of the polar bodies, and in the centrosomes.

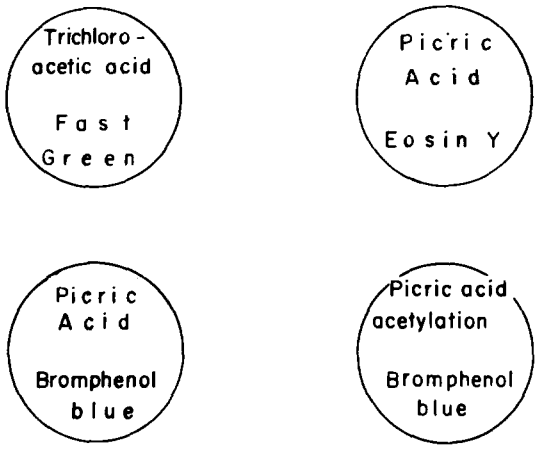
During the first four or five cleavage divisions, the interphase nuclei are large, roughly 20 $\mu$ in diameter. The chromatin forms a skein, connecting between ten and twenty large nucleoli (Fig. 11). These nucleoli stain darkly with bromphenol blue, and provide the most constant exception to the specificity of bromphenol blue for chromosomal proteins under these conditions. The chromatin stains very faintly with Feulgen, because of its diffuseness, and it is, therefore, not surprising that little if any staining with bromphenol blue is detectable. Condensed mitotic chromosomes do exhibit bromphenol blue staining, however. These same chromosomes remain unstained with the alkaline fast green method. In later development the nuclei are smaller, and now stain in interphase with bromphenol blue (Fig. 12) and remain unstained by alkaline fast green.

At a stage of development (probably gastrula, Fol (9)), about 48 hours after laying, the chromatin

again becomes fast green positive, indicating a return to a more "typical" histone (Figs. 13, 14). The mitotic chromosomes exhibit an intense fast green staining. The interphase nuclei stain less intensely because of the diffuseness of the chromatin; nevertheless, such staining is well above the limit of detectability and fast green provides, during these stages, a good nuclear stain. Many of the nuclei are large and very active, as evidenced by their large nucleoli. This "heterosynthetic" condition (4) does not result in an appreciable masking of histone staining however.

During this stage, the polar body nuclei also exhibit fast green staining. The chromosomes are now condensed, as though mitotic, and although similar morphologically to the postmeiotic polar




See illustrations on pages 72 and 73.

body chromosomes, their composition is apparently different, as suggested by their staining properties. The polar bodies after meiosis had reverted to interphase.

## DISCUSSION

The methods used for the staining of histones, the binding of acid dyes at high pH's, is a departure from the usual aims of histochemistry and cytochemistry of quantitativity and stoichiometry. This compromise is in the interest of specificity, for the histones contain the same constituents as are found in the rest of the cellular proteins, and any "quantitative" stain would, therefore, fail to differentiate a histone from other cellular proteins. Instead, the methods seek to take advantage of the stronger affinity of very basic proteins for anionic dyes, at pH's at which typical proteins have no such affinity. As a result, changes in staining properties are sometimes difficult to interpret. A decrease in staining intensity may be attributed to an actual decrease in histone, masking of basic groups of the histones, decrease in the basicity of histone, the latter either by changes in the relative ratios of strongly basic arginine to the less strongly basic lysine, or an increase in the acid residues, glutamic and aspartic acids. Nevertheless, the cytologist who depends upon the use of staining techniques to identify, and to an extent, to characterize the histones may find encouragement in the fact that the conditions affecting dye binding can be accounted for, and usually lie within his control, as is shown by the correspondence between the staining properties of the histones on filter paper, and the cells from which the histones were extracted.

The low stainability of the early cleavage nuclei and chromosomes in *Helix* characterizes the third histone transition encountered upon tracing the course of nuclear changes from spermatogenesis (See Fig. 15). The first two, the transitions from a "typical" somatic histone to an arginine-rich histone, then to a protamine, are fairly well defined by the correlation of staining behavior with amino acid composition. The third change reflects a decrease in the basicity of the histone although the reasons for this decrease in terms of amino acid composition await a chemical characterization of the protein. That the loss of fast green staining is probably not attributable to a masking effect is indicated by the similarity in the staining properties of the early cleavage chromosomes and the condensed male pronucleus, and the observation that loss of

FIGURES 5 and 6

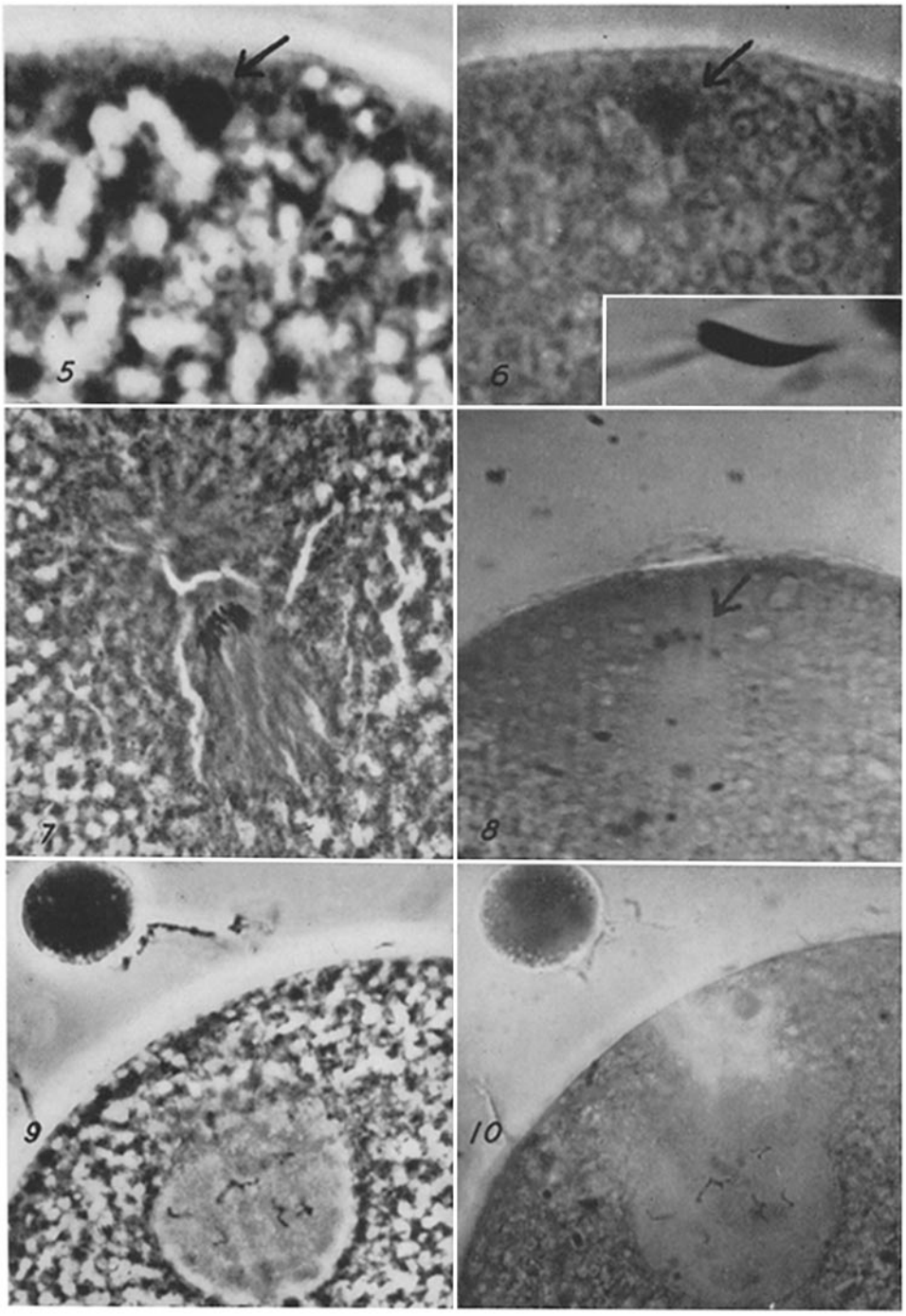
Phase and brightfield photomicrographs of an early male pronucleus stained with bromphenol blue. The cytoplasmic image in Fig. 6 is due to refractility. The staining of the male pronucleus is barely perceptible (arrow) as compared with the dense staining of the mature sperm (insert). All the brightfield photomicrographs of bromphenol blue-stained material were taken through a Wratten No. 22 filter. Magnification, 3,000.

FIGURES 7 and 8

Phase and brightfield photomicrographs of the second meiotic anaphase of two different eggs. The egg of Fig. 7 was stained with the Feulgen technique; that of Fig. 8, with bromphenol blue. The image of the cytoplasm in Fig. 8 is due to refractility. The chromosomes of Fig. 8 exhibit a faint but definite bromphenol blue staining. Magnifications, 1,200 and 1,000, respectively.

FIGURES 9 and 10

Phase and brightfield photomicrographs of the condensing chromosomes of the late male pronucleus. The chromosomes are faintly but definitely bromphenol blue-positive. The image in Fig. 10, apart from the chromosomes and the polar body, is due refractility. Magnification, 1,200.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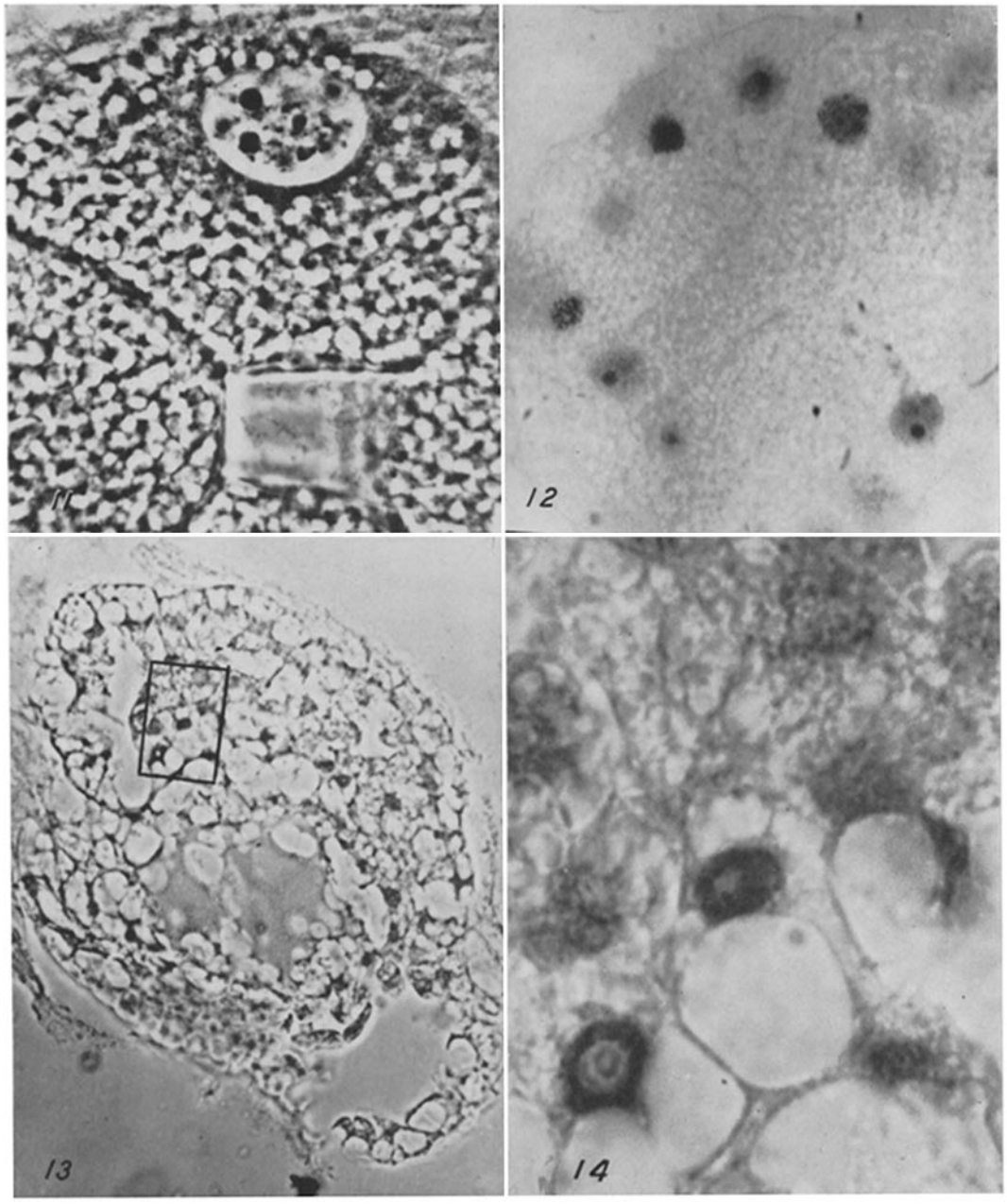
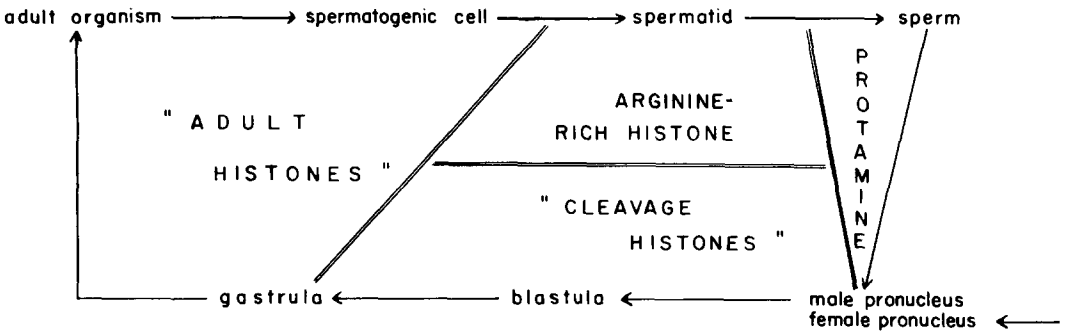
protamine staining in the male pronucleus is accompanied by a loss in the arginine content as demonstrated by use of the Sakaguchi test. A masking of histone staining, characteristic of "heterosynthetic" nuclei (4), may in fact occur during early cleavage; however, the large size of the nuclei, hence the dilution of chromatin, makes it impossible to decide whether or not an apparent lack of staining is due to masking and dilution, or merely to dilution. The characterization of the histone as one of low basicity is made possible by the observation that lack of fast green staining occurs also in condensed chromatin of the male pronucleus, the mitotic chromosomes, the meiotic chromosomes of the maturing egg, and the polar body chromosomes. Masking of staining might not






| | F G | HONO F G | B P B | EOSIN |
|---|---|---|---|---|
| "ADULT HISTONES" | + | − | + | + |
| ARGININE-RICH HISTONES | + | + | + | + |
| PROTAMINES | − | − | + | + |
| "CLEAVAGE HISTONES" | − | − | + | − |

FIGURE 15

Summary of the histone changes during the life cycle of *Helix aspersa* as followed through the male germ line.

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FIGURE 11

Phase photomicrograph of one of the four blastomeres of the cleaving egg. Bromphenol blue stain. The nucleoli in this section, as seen with bright field, exhibit a strongly positive reaction. The remainder of the nucleus is bromphenol blue–negative, possibly because of the diffuseness of the chromatin. Magnification, 1,100.

FIGURE 12

Brightfield photomicrograph of an egg prior to gastrulation, stained with bromphenol blue. Nucleoli and chromosomes stain strongly. The diffuse chromatin exhibits staining. The nuclei are fast green–negative at this stage. Magnification, 920.

FIGURE 13

Phase photomicrograph of a section through an egg fixed 48 hours after laying. Fast green stain. Magnification, 515.

FIGURE 14

Brightfield photomicrograph of an area of the section shown in Fig. 13, demonstrating the positive fast green reaction. The cytoplasmic image is due to refractility. Note the negative reaction of the nucleolus, which would be stained by bromphenol blue. Red filter. Magnification, 2,750.

be expected here because of the high concentration of chromatin and the apparent "shedding" of non-histone protein by the chromatin during stages prior to mitosis (4). (However, the sloughing of ribonucleoprotein from chromosomes during mitosis (13) and the recent demonstration of RNA in mitotic as well as interphase chromatin by Kleinfeld and Von Haam (14) make it unreasonable to rule out masking by non-histone proteins as an explanation of loss of fast green staining of mitotic chromosomes.)

Alfert has found that in mouse, the return to fast green positive staining among interphase cells occurs much earlier than these studies in the snail indicate. Furthermore, the mitotic chromosomes remain stainable with fast green. Hence he attributes fast green negativity to masking.

Alternative interpretations of the transition during development from a fast green negative to a fast green positive condition would be: (*a*) a gradual change in the histones, these proteins reaching an isoelectric point compatible with fast green staining during the gastrula stage, or (*b*) uniformity of the fast green negative histones, an abrupt change occurring in the gastrula. Consistent with the former hypothesis, would be a view of the fast green negative histones as covering a spectrum of substances, the range of the spectrum extending over developmental periods of the egg, rather than over different areas of the egg.

Whether differences exist among the fast green negative histones of the developing egg owing to "cell specificity" is uncertain. The abruptness of the transition to fast green positive, which occurs in most, if not all cells, including the polar bodies, suggests that the earlier histones may be characteristic of the entire egg, rather than of specific cells. Whether or not histones exhibit cell specificity remains one of the crucial questions regarding histone function.

The direction of a histone change, toward an increasing basicity, is in accord with the observation of Horn and Anderson, of an increasing histone-to-DNA ratio during the development of the chick embryo (11). Their study covered a developmental period much later than that concerned in the present experiment however. Of particular interest are the observations of Briggs and King, of a loss of totipotency of the nuclei during gastrulation in the frog embryo (6). The regulation or reflection of developmental potency by the nuclear histones remains an enticing possibility.

## SUMMARY

By correlating the staining behavior of histones with their amino acid compositions, it was found that various modifications of a standard method for demonstrating histones would permit characterization, *in situ*, of "typical" somatic histones, arginine-rich histones, protamines, and a weakly basic histone found in some cleaving eggs. These methods were applied to the developing egg of *Helix aspersa*. On fertilization, the protamine of the sperm is lost. The male pronucleus, and the maternal chromosomes of the egg, contain a weakly basic histone which is not stained by the standard fast green test, but which can be demonstrated using a more sensitive bromphenol blue procedure. The ability to bind bromphenol blue is retained during the later development of the organism. The ability to bind fast green is regained by the histones of most of the cells in the gastrula stage, approximately 40 to 48 hours after laying. The significance of this change is discussed.

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