EXTRANUCLEAR HISTONE IN THE AMPHIBIAN OOCYTE*

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Cellular basic proteins of relatively low molecular weight (ca. $8 \times 10^4$ to $3 \times 10^4$) combined with nucleic acids have been generally considered to be restricted to the cell nucleus. Indeed, the classical source of protamines is the sperm of various vertebrates; 1 while histones are conventionally obtained by extracting isolated cell nuclei with dilute mineral acids, the original method used by Kossel. 2

The development by Alpert and Geschwind 3 of a relatively simple procedure for visualizing the cytological distribution of basic protein in tissues has stimulated numerous studies designed to reveal the distribution and possible role of the histones in cells from many sources. 4–13 Until recently, the majority of all reports describing the distribution of histone substantiated the viewpoint that this class of proteins is restricted to the cell nucleus. In this laboratory, sections of various amphibian, avian, and mammalian adult tissues have been examined after treatment with the Alpert and Geschwind technique. In none of these have we discovered extranuclear staining indicative of basic protein.

It was with considerable interest, then, that we discovered, incidental to a study of basic protein localization in Drosophila salivary gland chromosomes, 4 a strongly positive alkaline fast green staining reaction by the larval salivary gland cytoplasm. In addition, this same cytoplasm also gave a strong Sakaguchi reaction indicative of the high arginine content characteristic of many histones. Because the results of the alkaline fast green test are a measure of the net charge in a particular protein species, a positive staining reaction can provide only an indication of high net positive charge such as is found in histones and protamines. Therefore, when a consistent positive staining reaction is obtained from a cell constituent, qualitative analyses more rigorous than the staining technique provides are ab-
olutely essential if any but the most general conclusions are to be reached concerning characterization and identification of a suspected protein component. Electrophoretic mobility, sedimentation rate, and amino acid composition of the suspect protein should be determined to aid in its identification. The number of salivary glands of Drosophila larvae necessary for such an analysis discouraged further work with that material.

A positive alkaline fast green staining reaction in cytoplasm was first reported\textsuperscript{4} for Tetrahymena. Although the responsible material seemed to be associated with RNA, the authors wisely avoided any statement of its nature. Cowden\textsuperscript{9} described a positive reaction in the cytoplasm of sectioned slug oocytes but chose to consider the observation an indication of the lack of specificity of the reaction. Taleporos\textsuperscript{10} reported positive alkaline fast green staining in the cytoplasm of sea urchin eggs. The reaction was such as to indicate disappearance of the staining material during early embryogenesis. Yield and solubility characteristics of material obtained by acid extraction of unfertilized eggs and a considerable lysine and arginine content in the acid hydrolysate of the extract suggested the presence of "histone."\textsuperscript{11} Butler, Cohn, and Simson\textsuperscript{14} have reported the likelihood that microsomes from rat liver cells contain basic proteins with a composition similar to that of nuclear histones. Their suggestion was based on data on amino acid composition and N-terminal group analysis of certain microsome protein fractions separated by the use of detergents.

Horn\textsuperscript{15} reported a strongly positive alkaline fast green reaction in the yolk platelets of amphibian oocytes and early blastomeres. In sharp contrast, the nuclei of oocytes and pre-gastrula cells gave a negative (colorless) reaction for basic proteins. At gastrulation, the nuclei of all cells first showed the typically positive, intense staining reaction of adult cell nuclei. The present report extends this original observation and characterizes the basic protein as histone.

\textbf{Materials and Methods.—General:} Mature oocytes were obtained from adult female Rana pipiens\textsuperscript{16} after pituitary injections. Some oocytes were manually oviposited and were fertilized in sperm suspensions. Samples of these zygotes, maintained at 18°C, were staged (Shumway) and fixed in 10% neutral formalin. Serial sections of all stages from oocyte to hatching were prepared.

Other pituitary-injected frogs were stunned, pithed, and opened mid-ventrally. Ovulated oocytes were washed from the coelom with 10% amphibian Ringer’s, pooled, and used for extraction.

Clutches of Ambystoma opacum embryos taken from ponds in Duke Forest were reared in the laboratory at 20°C. At regular intervals, groups of embryos were staged, fixed in 10% neutral formalin, embedded, and serially sectioned.

John R. Gregg provided us with hybrids of \textit{R. pipiens} ♀ × \textit{R. sylvatica} ♂ which were reared in the laboratory at 18°C and from which a series of early developmental stages were fixed for the purpose of comparing with the normal \textit{R. pipiens} series. Embryos of this hybrid appear to cease all gross morphological changes at the early gastrula stage.\textsuperscript{17}

All slides of embryonic material contained a section of adult frog liver for comparison and stain control.

\textbf{Staining procedures:} The alkaline fast green method of Alfert and Geshwind\textsuperscript{8} and the picric acid–bromphenol blue procedure of Bloch and Hew\textsuperscript{12} were used on selected stages of all sectioned material. Deamination with nitrous acid was carried out on selected stages of sectioned material and followed with acid bromphenol blue staining.\textsuperscript{13}

\textbf{Extraction of basic protein:} Because of the cytochemical evidence for localization of basic proteins in the yolk platelets, the initial extraction of coelomic oocytes was begun with the mass isolation of platelets by conventional centrifugal and density gradient techniques. Although enough
success was enjoyed to provide one large batch of moderately clean platelets for extraction, subsequent extractions omitted this step from the procedure. The characteristics of the crude extracted product with or without the initial isolation of platelets were insufficiently different to risk the extra time and consequent proteolysis which might otherwise have resulted. All subsequent extractions have been performed directly on the entire oocyte. Comparison of electrophoretic analysis of the products obtained in the 1960 and 1961 seasons has shown that the extraction method provides consistent results.

Batches (6 to 10 ml, 1 × g) of coelomic eggs were suspended in four times their volume of 0.1 N HCl and homogenized gently (four strokes of Potter-Elvehjem grinder). The resultant brei was centrifuged at 30,000 rpm for 90 min (RCF = 78,410).

The clear yellow supernatant solution was carefully separated from the pellet and then gradually alkalinized by the dropwise addition of 0.1 N NaOH with constant stirring by magnetic mixer. A voluminous flocculent precipitate occurred in the pH range of 3.5–4.0. After standing overnight at this pH, the suspension was centrifuged 30 min at 30,000 rpm (RCF = 78,410). Renewed addition of dilute NaOH to the separated supernatant solution produced a light, fine precipitate around pH 7. Centrifugal separation (RCF = 78,410 for 30 min) yielded a clear colorless supernatant. Protein analysis by Lowry's method indicated that this solution contained about 1% protein. Addition of more NaOH demonstrated that nothing precipitated from this solution until a pH of about 10 was reached. The precipitate produced at pH 10 was partially soluble in an excess of NaOH but insoluble in an excess of NH₄OH.

Products obtained from five separate oocyte extractions and soluble at pH 7 were individually dialyzed against several changes of distilled water and then pooled. The combined extracts represented roughly 5.5 × 10⁴ coelomic eggs obtained from 72 pituitary-stimulated female frogs. After centrifugation, the clear pool of dialyzed supernatants was frozen and lyophilized, yielding 1.8 grams of a product hereafter designated as the crude extract and used for all qualitative analyses.

All procedures were carried out in the cold (4°C).

Electrophoretic analyses: A Spinco Model H electrophoresis apparatus was used. Physical constants for the analyses are stated with the results.

Ultracentrifugal analyses: The ultracentrifugal analyses were obtained by Robert E. Canning with a Spinco Model E analytical ultracentrifuge equipped with rotor temperature indicator and control permitting measurement (±0.02°C) and control of rotor temperature during a run to within 0.1°C. All values given have been corrected to 20°C in water.

Amino acid analyses: The amino acid analyses were carried out by Robert Becker and Sally L. Miller with the aid of a Spinco Model 120 Analyzer.

Results.—Cytochemical observations: Serial sections of both R. pipiens and A. opacum embryos treated with alkaline fast green presented the same general staining reactions; hence, no distinction has been made in their description.

The yolk platelets of ovarian and coelomic oocytes as well as of cleavage blastomeres reacted positively to the stain. Because of the high concentration of platelets in the macromeres, these cells appeared to be stained more intensely. However, the staining of individual platelets varied in intensity in the oocyte and in both micromeres and macromeres of cleavage stages. In the micromeres of early embryos, the relatively few intensely staining platelets were grouped loosely about the nucleus. Platelets in cells of post-gastrula embryos also stained but variation in intensity was marked. Rapidly differentiating cells, e.g., those constituting the developing nervous system and musculature, contained platelets with little or no staining capacity, while platelets in the cells forming the yolk mass reacted most positively but still with individual variation.

The nuclei of these same embryonic cells reacted with alkaline fast green in a manner which was in sharp contrast to the cytoplasmic reaction. Nuclei of oocytes and of blastomeres through the blastula stage did not stain. At the onset of gastrulation, however, the nuclei of all cells including those forming the yolk mass
reacted positively with alkaline fast green, completely resembling similarly treated nuclei in adult tissues. Throughout gastrulation and subsequent stages to hatching, the nuclei continued to give an unaltered positive reaction.

Interestingly enough, the fast green staining reaction of sections from hybrid embryos was indistinguishable from that of the *R. pipiens* series. Of course, no tissue comparisons could be made beyond the gastrula stage because the hybrid exhibits gastrula-arrest, but the same lack of nuclear staining existed in all pregastrula hybrid cells and the same rather abrupt alkaline fast green staining of the nucleus occurred concurrently with the first gastrulation movements. In all stages, the yolk platelets of hybrid cells stained with an intensity comparable to those of *R. pipiens* cells.

Examination of material treated with the picric acid–bromphenol blue procedure revealed essentially the same staining pattern as was obtained with fast green. One noteworthy exception was shown by pregastrula nuclei. The chromosomes of mitotic stages of pregastrula nuclei stained very faintly; interphase nuclei not at all. Here again, the results were indistinguishable in material from *Ambystoma*, *R. pipiens*, and the hybrid.

When sections which had been hydrolyzed with picric acid and deaminated with nitrous acid to block lysine staining were subsequently treated with acid bromphenol blue to reveal arginine-rich histone, only the yolk platelets stained and then much more weakly than the nondeaminated controls. Again individual platelets varied in intensity of staining reaction. This same result, i.e., lack of nuclear staining, weak platelet staining, was obtained regardless of developmental stage whenever deamination preceded the stain. The adult frog liver sections used as controls were completely unstained except for the nuclei of erythrocytes and for some large extranuclear granules in an undetermined cell type. Both of these exceptions stained brightly after deamination.

*Ultracentrifugal analyses:* Ultracentrifuge data revealed three distinct peaks with sedimentation rates (*S*₂₀,ₐ) of 5.78, 3.87, and 1.62. The lowest value indicates a molecular species well within the range of known histones.

*Electrophoretic analyses:* Figure 1 shows the electrophoretic pattern obtained from a 1.6 per cent solution of the crude extract in Miller-Golder¹⁸ phosphate buffer after 120 min. The initial boundary position is marked by the arrow; electrode polarity by conventional symbols. The initial buffer-protein boundaries were moved approximately one third the length of the cell channel to permit simultaneous detection of proteins with net positive or negative charge. The pH was selected below the isoelectric point of known histones to maintain these proteins as cations and yet above or near the isoelectric point of most larger known proteins found in animal systems, where they would behave as anions.

Figure 1 shows that at least six boundaries were identifiable, one of which (marking the highest concentration) is the concentration boundary. At least three peaks represent materials which have migrated cationically, indicating their basic nature. Their mobilities have been calculated and are presented in the notation of Anderson *et al.* (ref. 19), \( \text{pHos}_{0.3} T_{7.5} \), ¹⁰ to indicate that the studies were conducted in a phosphate buffer, 0.1 ionic strength, at 0.9°C, at pH 7.5. A plus sign indicates that the protein moved toward the cathode:

\[
\begin{align*}
(1) \ T_{7.5} & = +7.97, \\
(2) \ T_{7.5} & = +5.61, \\
(3) \ T_{7.5} & = +2.79.
\end{align*}
\]
FIG. 1.—Electrophoretic pattern of oocyte acid extract after 7,200 seconds in pH 7.5; ionic strength, 0.1; phosphate-NaCl buffer. Arrow marks starting boundary. Cathode indicated by minus sign.

**Amino acid analyses:** Table 1 presents the amino acid analyses of the crude extract after 24 and 60 hr hydrolysis. Although the proportion of lysine and arginine present was not as high as that which has been reported for isolated histones,

<table>
<thead>
<tr>
<th></th>
<th>24-hr hydrolysis</th>
<th>60-hr hydrolysis</th>
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<tbody>
<tr>
<td></td>
<td>µg N\textsubscript{1}</td>
<td>% N\textsubscript{1}</td>
</tr>
<tr>
<td>Lys</td>
<td>9.74</td>
<td>12.6</td>
</tr>
<tr>
<td>His</td>
<td>5.42</td>
<td>7.0</td>
</tr>
<tr>
<td>NH\textsubscript{3}</td>
<td>8.08</td>
<td>10.5</td>
</tr>
<tr>
<td>Arg</td>
<td>9.46</td>
<td>12.3</td>
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<tr>
<td>Asp</td>
<td>6.24</td>
<td>8.1</td>
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<tr>
<td>The</td>
<td>3.26</td>
<td>4.2</td>
</tr>
<tr>
<td>Ser</td>
<td>2.62</td>
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<td>Glu</td>
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<td>Ala</td>
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<tr>
<td>1/2 Cys</td>
<td>3.92</td>
<td>5.1</td>
</tr>
<tr>
<td>Val</td>
<td>2.98</td>
<td>3.9</td>
</tr>
<tr>
<td>Met</td>
<td>0.83</td>
<td>1.1</td>
</tr>
<tr>
<td>Ile</td>
<td>2.23</td>
<td>2.9</td>
</tr>
<tr>
<td>Leu</td>
<td>2.30</td>
<td>3.0</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.14</td>
<td>2.8</td>
</tr>
<tr>
<td>Phe</td>
<td>2.02</td>
<td>2.6</td>
</tr>
<tr>
<td>Total µg N\textsubscript{2}</td>
<td>76.98</td>
<td>76.37</td>
</tr>
<tr>
<td>Per cent recovery</td>
<td>109.9</td>
<td>109.1</td>
</tr>
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it should be recalled that the ultracentrifugal and electrophoretic analyses indicated that the crude extract contained at least two components which did not correspond to the classical definition of a histone. Presumably, these congeners would serve to lower the observed relative proportions of the lysine and arginine provided by the histones present.

Discussion.—The present studies obviously provide no data which might be used to determine the role of extranuclear histone in development. However, a more exact localization of this material within the platelet may be conjectured from Karasaki's recent electron microscopic studies of developing nucleus and cytoplasmic structures in embryonic _Triturus_ cells. He has shown that the yolk platelets of presumptive ectoderm cells prior to gastrulation consist of a homogeneous compact central body, comprising most of the platelet. Surrounding this core is a superficial layer of fine particles measuring about 10 μ in diameter. At this early stage, the platelet is bounded externally by a single membrane about 6 μ in thickness. The further developmental history of these platelets reveals that the external membrane and the superficial granular layer disappear during the early phases of differentiation. The dense core, on the other hand, remains essentially unchanged through the early tail bud stage. Because the morphological disappearance of the granules coincides temporally with the lessening in basic protein stain intensity shown by this study, it seems reasonable to suggest that basic protein comprises a portion of these granules. Indeed, on morphological grounds Karasaki considered them to be ribonucleoprotein particles of the type characterized by Palade and Siekewitz. The presence of RNA in yolk platelets has been reported several times, and at the less than neutral pH which prevails in amphibian embryonic cells, histones would act as polyvalent cations combining in salt linkages with phosphoric acid groups of nucleic acid. One is reminded immediately of the work of Butler _et al._ who demonstrated the presence of histone-like proteins in the RNA-rich microsomes from rat liver cells. However, Yamada has cast considerable doubt on the presence of appreciable amounts of RNA in the superficial layer of yolk platelets. He cites the unpublished work of Takata and Ono, whose cytochemical studies not only indicate an absence of RNA but suggest the presence of an acid polysaccharide in this layer. In any case, the observed lessening of the yolk platelet reaction to fast green staining indicates reduction in quantity of platelet basic protein whether the protein be associated with RNA, acid polysaccharide, or other substances. This same reduction coincides with the disappearance from the platelets of their superficial granule layer described by Karasaki.

During amphibian development, detectable RNA synthesis does not begin until gastrulation, nuclear RNA requirements apparently being met at least in part by transfer from the cytoplasmic reserve. Although one might more readily concede a fabrication of nuclear RNA from a ribonucleoside and ribonucleotide pool, an existent mechanism for a direct transfer of ribonucleoprotein to the nucleus has been demonstrated morphologically. Karasaki's studies (op. cit.) also showed that during gastrulation invagination of the nuclear surface occurred, trapping cytoplasm, including its microparticles, in extensive intranuclear pockets. In addition, the nuclear envelope at this stage is provided with pores having a mean diameter several times that of the granules which form the platelet peripheral layer. One
cannot, in the face of this evidence, escape the possibility of in toto microparticle transfer to the nucleus from the cytoplasm.

The relatively abrupt change of nuclear stain intensity which occurs at gastrulation is at once startling and provocative. The same observation has been reported for the same stage in snail development by Bloch and Hew. 13 As they pointed out, the phenomenon might reflect a dilution effect and/or a masking of the proteins, the two conditions possibly operating together in unknown degrees. On the amphibian material of this study, the disparity in nuclear volumes of either blastula or gastrula cells from different regions of the embryo would provide a range of basic protein concentrations per nucleus such that the staining reaction would be correlated with nuclear volume rather than with developmental stage. Such did not seem to be the case. As pointed out in the Results, the larger nuclei of the yolkladen cells and the smaller presumptive ectodermal cell nuclei first reacted positively to fast green at the same stage. Furthermore, Ambystoma material first stained positively by stage rather than by nuclear volume. These observations suggest that a dilution effect plays at best a minor role in the staining reaction of pre- and post-gastrula cells. The deamination studies indicate that this sudden refulgence is due at least in part to lysine-rich basic protein. Temporal coincidence between the change in nuclear staining and possible transfer of microparticles to the nucleus from the cytoplasm suggests RNP as a possible amino acid source or complete source of the nuclear protein responsible for the altered staining pattern.

The studies on reciprocal androgenetic amphibian hybrids by B. Moore 28 and particularly the clonal (back transfer) nuclear transplantation experiments by King and Briggs 29 have forced recognition of the importance of cytoplasmic factors in the production of irreversible and progressively restrictive changes of the nucleus. Unfortunately, this revelation loses some of its lustre when one soberly considers that DNA, 27, 28, 30–32 RNA 22, 23, 27, 32 and now histone have all been identified in the cytoplasm of the amphibian egg. At one time or another, each of these three familiar nuclear substances has been especially implicated in the determination of cell specificity.

The occurrence of DNA in the cytoplasm of amphibian oocytes together with its apparent genetic and metabolic importance naturally make it an attractive material about which to design hypothetical explanations for differentiative processes. Thus, J. Moore 34 has demonstrated that the blastula nuclei of androgenetic hybrids have undergone irreversible changes by virtue of their residence through cleavage stages in the cytoplasm of another species. After such a passage, they are incapable of supporting development beyond the gastrula stage when placed in an enucleated egg of their own species. He has tentatively suggested that this incapacity might be due to the transfer of preformed DNA-like substances from the cytoplasm of the egg to the resident nucleus of the other species. The resultant chromosomes "might not then replicate in a genetically normal fashion" even when transferred back to their original species-specific cytoplasm.

Attractive as such an hypothesis may be, alternative explanations for irreversible nuclear differentiation should be sought and tested. As early as 1954, Cruft, Mauritzen, and Stedman 35 suggested that nuclear histones might be species-specific and might function as gene inhibitors. Recently, Leslie 36 has gathered together new information which documents and extends the concept of histones as
gene regulators. His hypothesis includes postulating two forms of histone, one located in the nucleus to suppress selectively the template function of nuclear RNA, the other cytoplasmic (ribosomal) to protect template RNA and as ribonucleases to suppress by depolymerization RNAs of different ("wrong") base composition.

Leslie does not attempt to prescribe in the terms of his hypothesis the conditions existing in an oocyte nucleus or in the nuclei of early cleavage products. The work of Briggs and King\textsuperscript{27, 28} has demonstrated that these nuclei (providing they have not been exposed to gastrula or post-gastrula cytoplasm) are capable of supporting essentially complete development. Therefore, special conditions must prevail in pregastrula nuclei, limiting conditions of a kind which prevent the premature expression of the genome, i.e., the cellular production of any specialized product which might be interpreted as differentiation. One of the simplest types of limits which could be postulated to fulfill the conditions would be the absence from the nucleus of specific substances necessary to synthetic activity.

If the presence of nucleolar material is in some way a measure of RNA synthesis, then the observed absence of nucleolar material from the nuclei of amphibian blastomeres prior to the blastula stage would suggest that these nuclei simply do not possess RNA-synthesizing capacity. This suggestion has already been made by B. Moore\textsuperscript{29} who called attention to the absence of nucleoli in the cells of arrested blastulae of certain androgenetic hybrids. It seems quite possible that pregastrula nuclei may be unable to synthesize template RNA until after the basic proteins of the yolk platelets take up residence in the nucleus during the late cleavage or blastula stage. This transfer and its possible implications become all the more interesting when considered together with the cytological reports by Witte\textsuperscript{30} for amphibian oocytes and by Yamamoto\textsuperscript{40} for sh ioocytes that nucleolar material is released to the cytoplasm from the nucleus during oogenesis. This concept of early basic protein transfer to the nucleus together with that of Leslie would provide an explanation for hybrid developmental arrest quite independent of the transfer and incorporation of DNA or DNA precursors from cytoplasm to nucleus.

Summary.—Basic proteins have been demonstrated in the yolk platelets of amphibian oocytes. Their sedimentation rates, mobilities, and amino acid composition place them in the broad class of histones.

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† Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.
\textsuperscript{2} Kossel, A., \textit{Z. Physiol. Chem.}, 8, 511–515 (1884).
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\textsuperscript{8} Horn, E. C., and C. L. Ward, these \textit{Proceedings}, 43, 776–779 (1957).
\textsuperscript{10} Taleporos, P., \textit{J. Histochem. Cytochem.}, 7, 322 (1959).
METABOLIC PROPERTIES OF A RIBONUCLEIC ACID FRACTION IN YEAST*

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In a previous communication, Ycas and Vincent1 reported that if exponentially growing yeast is presented with a short pulse of orthophosphate, $P^{32}$, the ratios of total counts in the 2' and 3' ribonucleotides obtained by alkaline hydrolysis of the RNA are similar to the ratios of the corresponding deoxyribonucleotides in yeast DNA (uracil being regarded as corresponding to thymine). In exponentially growing yeast, labeled phosphorus initially enters an unstable RNA fraction having a composition similar to the corresponding DNA. Volkin, Astrachan, and Countryman3 had demonstrated earlier that such a fraction exists in phage-infected bacteria, where newly formed RNA appears to mimic the composition of phage DNA. Subsequently, other workers have reported the existence of similar RNA fractions