# ELECTRON MICROSCOPY OF THE AMMONIACAL SILVER REACTION FOR HISTONES IN THE ERYTHROPOIETIC CELLS OF THE CHICK

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## ABSTRACT

The product of the postformalin ammoniacal silver reaction, which has been claimed to distinguish lysine-rich from arginine-rich histones with the light microscope on the basis of a color difference, was examined in developing erythroid cells of chick bone marrow with the electron microscope. Stem cells and early erythroblasts exhibit no, or little, ammoniacal silver reaction product, while small basophilic erythroblasts, polychromatophilic erythrocytes, and reticulocytes exhibit an increasing amount of reaction product as maturation proceeds. The reaction product is in the form of discrete electron-opaque particles associated with heterochromatin. The ammoniacal silver reaction in the erythroid cell series is interpreted as reflecting either the accumulation of newly synthesized arginine-rich histones or changes in the availability of reactive sites in preformed histones.

## INTRODUCTION

Interest in histones has broadened since 1950 when Stedman and Stedman (30) suggested that these basic proteins may function as gene modifiers. Since then, more specific biochemical evidence has implicated the histones in possible repression of DNA-dependent RNA synthesis. Investigations of Huang and Bonner (16) and Allfrey, Littau, and Mirsky (2) have shown that arginine-rich histones differ from lysine-rich histones in their relative capacity to inhibit messenger RNA synthesis.

A staining reaction using postformalin ammoniacal silver to demonstrate qualitative color differences for nucleohistones from several sources was developed by Black and Ansley (5); a yellow stain was reported as characteristic of lysine-rich histones and a brown-black stain, characteristic of arginine-rich histones. Such staining differences

were detected in isolated histone extracts and with the individual amino acids.

The presence of histones was investigated by the authors (22) using the ammoniacal silver reaction in highly specialized cells, the developing erythroid cells of the chick bone marrow, which are known to shut down their nuclear RNA synthesis and the cytoplasmic synthetic machinery during maturation (7, 13, 18, 28). Observations on these cells showed the ammoniacal silver reaction as a yellow nuclear stain in early erythroblast cells and an increasing amount of brown nuclear stain in progressively more mature cell stages (22).

To study this reaction further and to determine whether the yellow coloration may be a dilution of the brown stain, we examined the results of the reaction with the electron microscope.

The reaction product was observed as a deposit

of electron-opaque particles in the nuclei of developing cells and correlated with the brown stain under the light microscope. A preliminary report of these observations has been presented (21).

## MATERIAL AND METHODS

Pieces of bone marrow removed from femurs of 3–12-day-old chicks were fixed for 3 hr at room temperature in 10% formalin neutralized with sodium acetate (2 g/100 ml of formalin) at pH 6.8–7.1. The tissue was thoroughly washed in distilled water for about 20 min before immersion in the ammoniacal silver solution (AS). The latter is prepared just before use by gradual addition of 10% silver nitrate solution to concentrated ammonium hydroxide until a slight turbidity persists. Tissues were immersed in the AS with no further agitation for 5 min. Another thorough washing in distilled water followed the AS treatment. Tissues were then placed for 5 min in 3%

formalin solution, at which point a yellow-to-brown coloration appeared. Tissues were washed in distilled water again and dehydrated immediately or stored in buffer at 4°C. (0.1 M sodium cacodylate was used before dehydration). Subsequent treatment included exposure to increasing concentrations of ethyl alcohol, propylene oxide, and final embedding in Epon 812. Curing overnight was done at 60°C. Thick sections (1–3 $\mu$) were cut with glass knives and examined under the light microscope without further staining. Thin sections were cut with a diamond knife on a LKB Ultrotome III or a Porter-Blum MT-2; these sections were stained with uranyl acetate, alone or followed by lead citrate, or left unstained before viewing with the Hitachi UH 11A electron microscope.

The ammoniacal silver method, like most metallic-impregnation methods, is capricious. Great care must be taken to use chemically pure reagents and clean glassware, and to avoid metal instruments in handling tissues. Ammoniacal silver solutions should be prepared immediately before use and the unused reagent
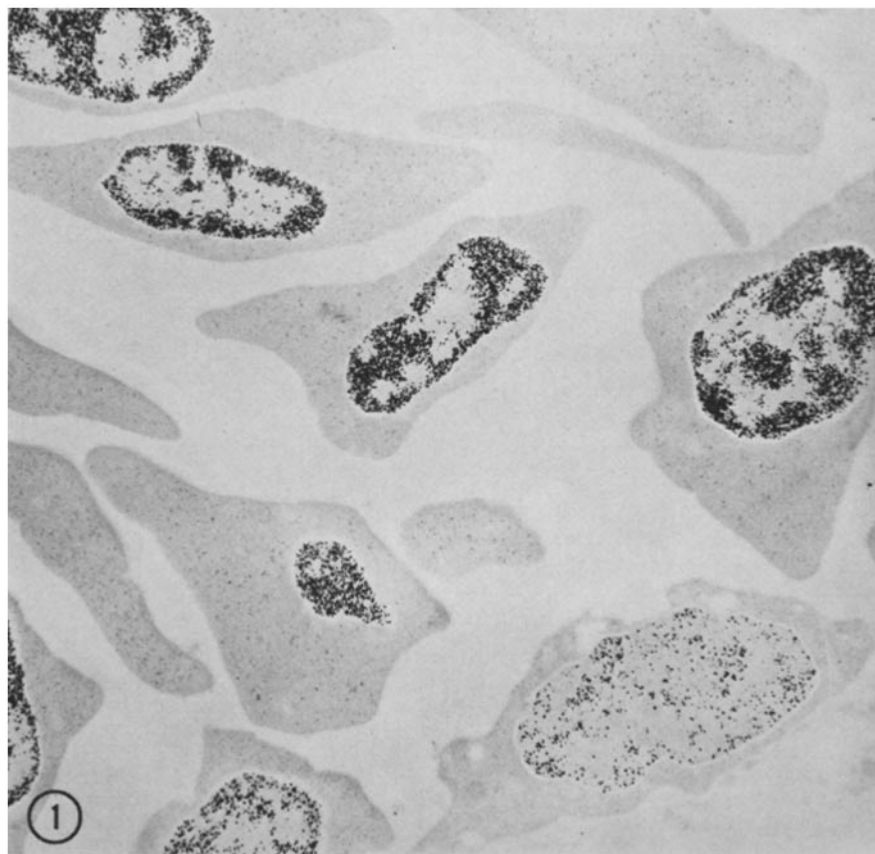


FIGURE 1  Low magnification of bone marrow cells after the ammoniacal silver reaction. Note the intracellular localization of ASR deposit in nuclear areas and a fine precipitate dispersed in the cytoplasm. × 9,000.

should be inactivated by excess dilute hydrochloric acid or a solution of sodium chloride, and discarded. Attention must be made to the potentially explosive properties of ammoniacal silver solutions, especially those stored for some time, exposed to sunlight, or prepared in well silvered flasks (31).

## OBSERVATIONS

The results of the application of the ammoniacal silver reaction (ASR) viewed with the electron microscope show a deposit of discrete electron-opaque particles localized in the nuclei of a variety of cells examined in the chick bone marrow (Fig. 1). The deposit, presumably a complex of silver, consists of particles, 190–200 A in diameter, occurring individually or in aggregates up to 500 A in diameter. Since it was necessary to stain the sections with uranyl and lead ions to provide

sufficient contrast for cellular identification, sections untreated with uranyl and lead ions served as control to check that the observed deposit was, indeed, due to the AS reaction and not to some other combination of metallic ions. Such controls show a particulate deposit confined intracellularly (Fig. 1).

Cells of the erythrocytic series in progressively maturing stages were identified by several characteristics, including an increase in nuclear density with clumping or condensation of chromatin (heterochromatin), a decrease in volume ratio of nucleus to cytoplasm, a decrease in cell size, and a change in cell shape. After treatment with uranyl and lead ions, ribosomes become visible as shown in Fig. 2. At higher magnifications, the difference in the number and cluster size of cyto-
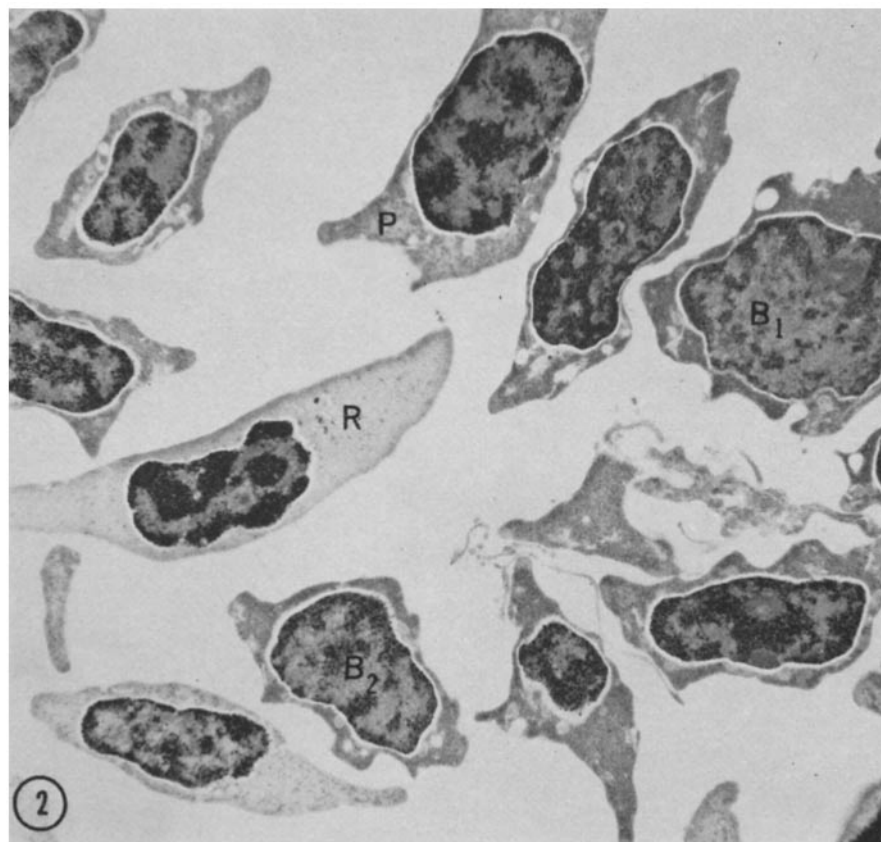


FIGURE 2   Low magnification of bone marrow cells of the erythroid series after ASR, uranyl, and lead ion treatment. The visualization of cytoplasmic ribosomes after uranyl and lead ions allows for identification of cells at different stages of maturation in this and subsequent micrographs. Large basophilic erythroblast $(B_1)$, a small basophilic erythroblast $(B_2)$, a polychromatophilic erythrocyte $(P)$, and a reticulocyte $(R)$. × 8,000.

plasmic ribosomes and in the number of mitochondria also serve as criteria for identification of cell stages. All subsequent micrographs are of cells treated with uranyl and lead ions after the ASR. The following classification of cells of the erythrocytic series, modified after the terminology of Lucas and Jamroz (20) is used: large and small erythroblasts, early, mid-, and late polychromatic erythrocytes, reticulocyte, and mature erythrocyte.

Stem cells, antecedent to the erythroblasts, were occasionally observed; they may be characterized as having a large, almost spherical nucleus filled with uniformly dispersed or extended euchromatin. Little or no evidence of chromatin condensation or clumping is seen at this stage.

Nuclear pores and nucleoli consisting of granular and amorphous material may be observed. Numerous ribosomes are closely packed throughout the cytoplasm. The lack of osmium tetroxide postfixation results in less contrast, particularly of the membranous organelles; nevertheless, mitochondria and sparse endoplasmic reticulum can be recognized. Mitochondria whose pale appearance resembles negatively stained images are round or rod-shaped with slender cristae extending the length of the mitochondria. A large cell identified as a possible stem cell or a very early basophilic erythroblast is seen in Fig. 3. Such cells show essentially no deposit of ASR in the nuclei.
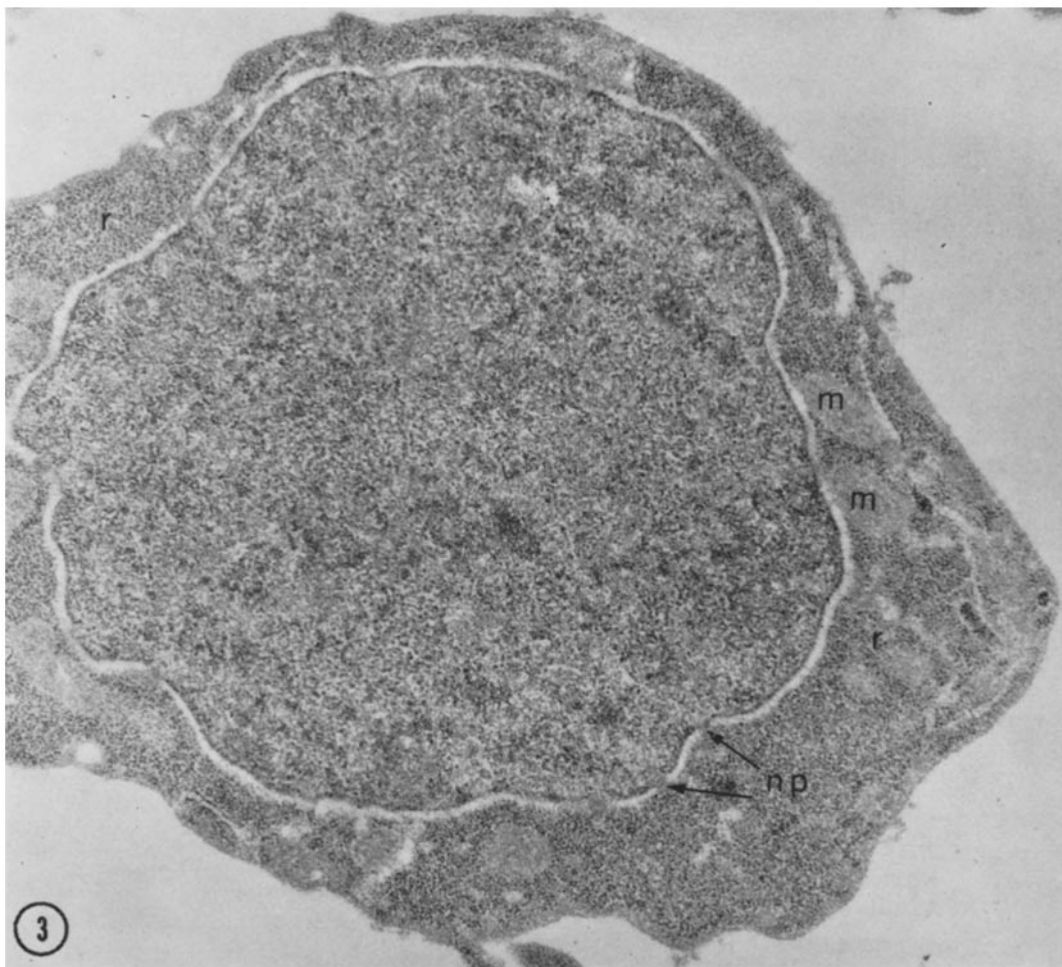


FIGURE 3  A stem cell or early basophilic erythroblast after treatment as in previous micrograph. The cytoplasm contains mitochondria (m) and closely packed ribosomes (r). Note the large nucleus and its homogeneous euchromatin, nuclear pores (np), and an absence of ASR particles. X 24,000.

The early large erythroblasts are distinguished from stem cells primarily by an increase in nuclear condensation indicated by areas of heterochromatin. The cytoplasm contains closely packed ribosomes and mitochondria similar to those of stem cells. The granular endoplasmic reticulum is sparse and occasional vacuoles appear in the cytoplasm. Some ASR deposit can be observed associated with the chromatin areas of increased density (Fig. 4). The nucleolus of such a cell is apparent and ASR particles appear primarily associated with the perinucleolar heterochromatin rather than with the nucleolus.

A small basophilic erythroblast is seen in Fig. 5; in this stage the cytoplasmic ribosomes are seen in clusters of polyribosomes (polysomes), and electron-lucent areas presumably containing hemoglobin become apparent around the poly-

somes. The ASR deposit is associated with heterochromatin in the periphery of the nucleus and perinucleolar areas.

The polychromatophilic erythrocyte stages can be distinguished from erythroblasts by several criteria. The volume ratio of nucleus to cytoplasm is decreased; there may be a decrease in cell size and further elongation of the cell. The nuclei of the polychromes display further chromatin condensation, and nucleoli are still present. The cytoplasmic ribosomes in the polysome arrangement are further dispersed and surrounded by more extensive areas of electron-lucent cytoplasm. Mitochondria are smaller and fewer in number. Nuclear pores are evident but somewhat fewer in number than in earlier stages. Bridges containing material of the same density and continuous with euchromatin extend from the nuclear pores into
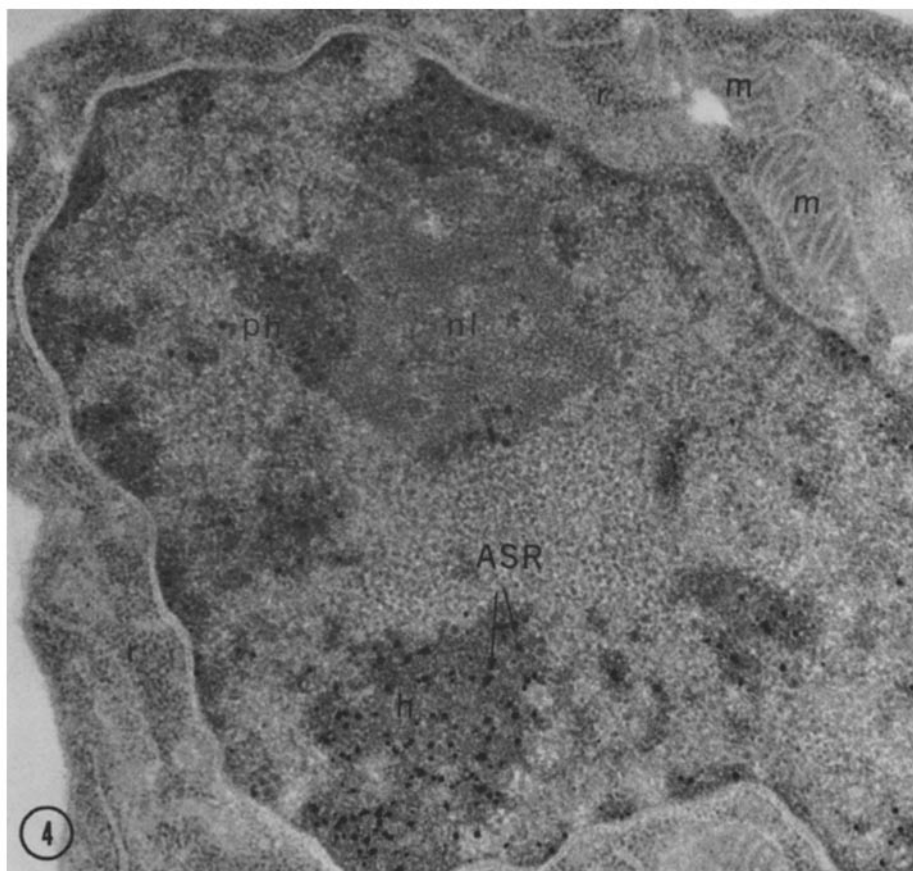


FIGURE 4   A part of a basophilic erythroblast with cytoplasmic ribosomes (r) and mitochondria (m). The nucleus has areas of heterochromatin (h) and associated ASR particles (ASR). The nucleolus (nl) is relatively devoid of ASR deposit as compared to the perinucleolar heterochromatin (ph). X 68,000.
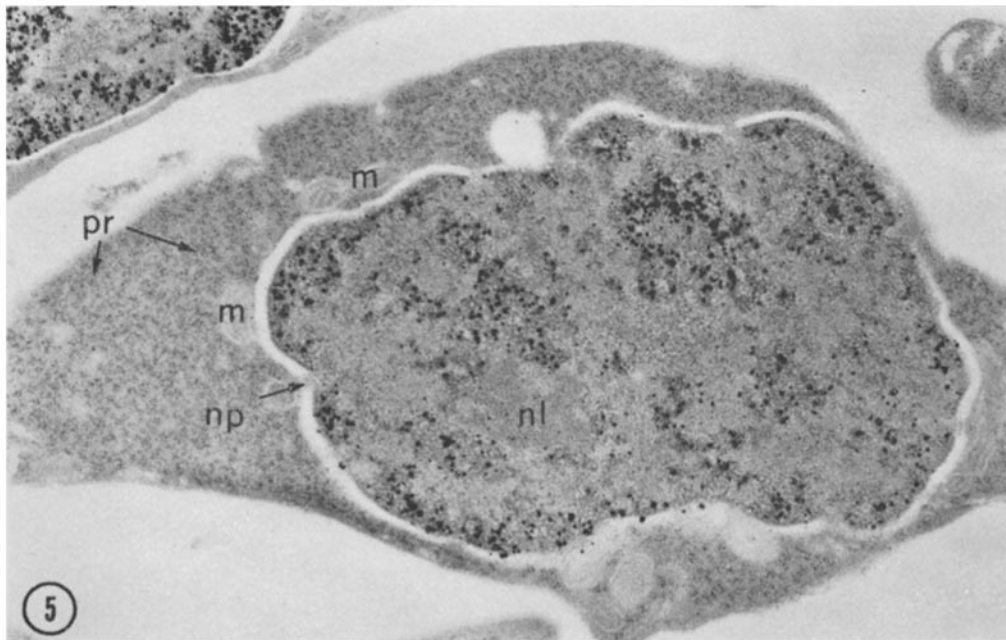
FIGURE 5   A small basophilic erythroblast contains cytoplasmic ribosomes arranged in clusters as polyribosomes (pr) and mitochondria (m). Note the ASR deposit associated with heterochromatin; the nuclear pores (np) and nucleolus (nl) are devoid of ASR deposit. × 23,000.
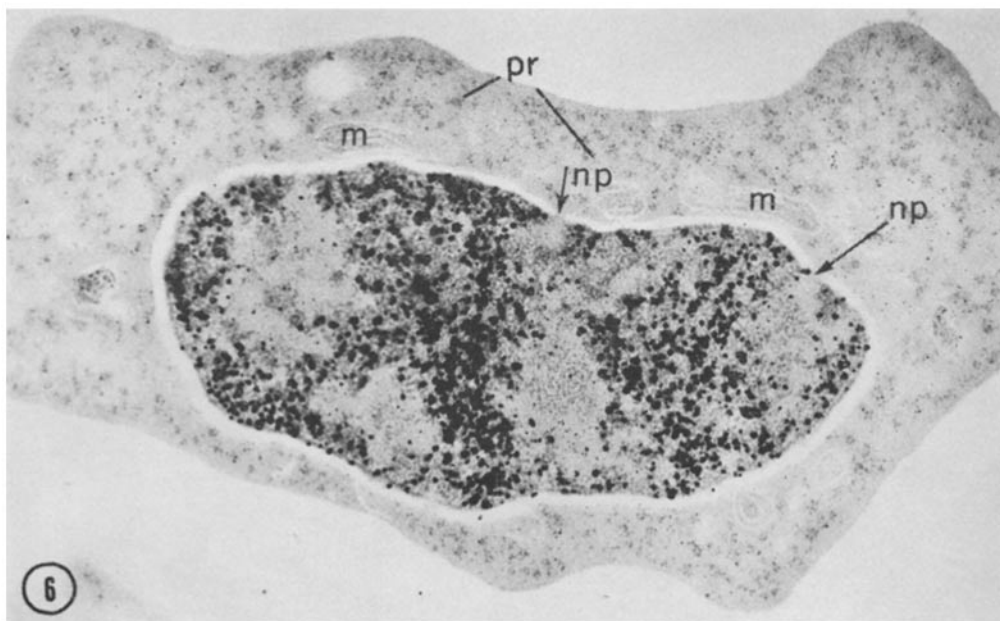


FIGURE 6   A polychromatophilic erythrocyte is shown with the increase in electron-lucent areas between the individual polysomes (pr) due to cytoplasmic accumulations of hemoglobin. Mitochondria (m) are still evident. Note the nuclear pores (np) and the increased amount of ASR deposit associated with heterochromatin. × 26,000.

Figure 7  A reticulocyte is shown with a smaller nucleus and a decreased number of polysomes as compared with earlier stages. Note the fine ASR precipitate evident in the cytoplasm. The nucleus has areas of heterochromatin heavily covered with ASR deposit. × 18,000.
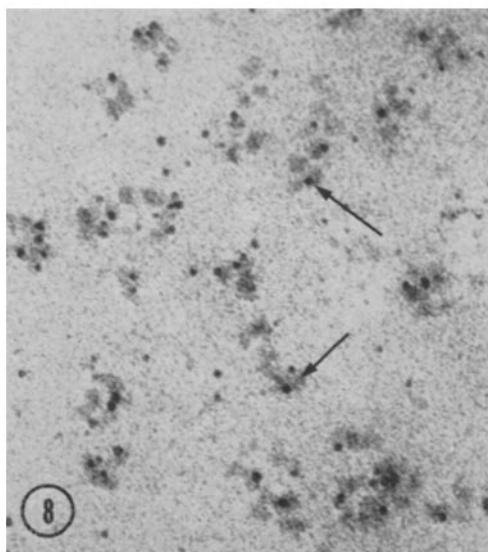


Figure 8  A higher magnification of the cytoplasmic polyribosomes of a late polychromatophilic erythrocyte. At arrows are indicated ASR particles associated with ribosomes. × 100,000.

the cytoplasm across a pale rim surrounding each nucleus. This rim may be due to shrinkage. A midstage polychrome is seen in Fig. 6. Closely associated with the increased amount of heterochromatin is the ASR deposit, also increased in amount and somewhat coarser in particle size.

The late polychromatophilic erythrocytes and reticulocytes have smaller volume ratio of nucleus to cytoplasm and show further condensation of chromatin into heterochromatin. Nucleoli are not observed. The number of ribosomes is decreased, and all ribosomes appear in the form of polysomes. Electron-lucent areas surrounding the polysomes are more extensive. The usual cytoplasmic density of hemoglobin-filled reticulocytes as seen after osmium tetroxide fixation is not observed in these formalin-fixed preparations. Few mitochondria are observed. The cell contours in late stages are relatively smooth. The clumped chromatin occupies a larger area in a smaller nucleus, and most of the heterochromatin is covered with ASR particles. A reticulocyte is shown in Fig. 7.

Mature erythrocytes have no polysomes or mitochondria in the cytoplasm. In comparison with reticulocyte stages, erythrocyte nuclei may be decreased in size but the extent of any further chromatin condensation and of ASR deposit is small.

In addition to the coarse ASR particles associated with heterochromatin and corresponding to the brown-black stain seen with the light microscope, there is present a fine precipitate of particles 40–80 A in diameter, observable in the cytoplasm. These particles seem to be present at all stages (see Fig. 1). However, in stained sections of the later stages in which polysomes are dispersed, the small particles appear to be associated with the ribosome clusters (Fig. 8).
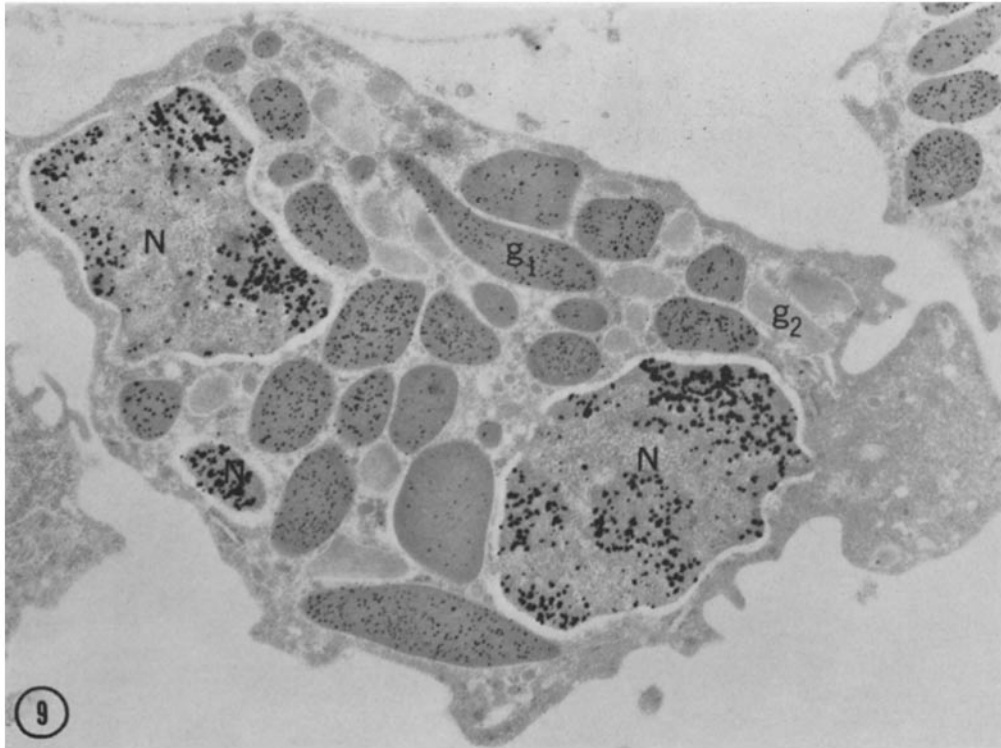
FIGURE 9   A leukocyte of the chick bone marrow after the ASR, uranyl, and lead ions. The ASR coarse particles are seen in the trilobed nucleus (N), and fine particles are seen in the larger and more electron-opaque secretory granules (g₁) but are lacking in the other granules (g₂). × 18,000.

Developing leukocytes present in the bone marrow also have ASR particles. One such leukocyte (heterophil) is seen in Fig. 9 which shows ASR particles associated with the heterochromatin of the polymorphic nucleus and a fine ASR deposit of 40–50 A in the larger and more electron-opaque secretory accumulations.

DISCUSSION

These observations have two implications: one regarding the ammoniacal silver reaction, and the other regarding the possible role of histones in cell development.

The ammoniacal silver reaction (ASR) particles seen with the electron microscope appear to correspond to the brown-black nuclear stain seen with the light microscope in the more mature stages of erythroid cell development. In contrast, the yellow nuclear stain seen with the light microscope in the early erythroblast stages has no reaction product detectable with the electron

microscope. The small number of ASR electron-opaque particles detectable in early basophilic erythroblasts are too few to be observable as a brown stain in the light microscope. The brown stain in localized nuclear areas of intermediate cell stages corresponds to deposit associated with heterochromatin in these cells. The chemical basis for the reaction is not known. Black and Ansley (5) suggested that the yellow stain may be due to ε-amino groups of lysine, while the brown-black stain may be due to guanidino groups of arginine.

One possible interpretation of this staining phenomenon as observed with the electron microscope is that the ASR is due to some specific interaction of silver with reactive centers in arginine and is detectable only in proteins with a high content of arginine. This category would include the arginine-rich fractions III, IV, and fraction V present in avian erythrocytes (23, 24). This interpretation assumes that a synthesis of

these arginine-rich fractions begins at about the erythroblast stages.

Another interpretation is that some structural rearrangement or reorientation of the histones makes previously masked reactive sites available to the ASR. This rearrangement would be presumed to occur at the erythroblast stages. Biosynthetic changes within the histone molecule, having an influence on DNA-histone structure as well as on DNA-dependent RNA synthesis, have been reported. Acetylation of certain residues in histones produces such an influence in early stages of cell differentiation in lymphocytes (25). The high peak of acetylation of the arginine-rich histones has been shown to occur before the maximal rates of RNA synthesis are reached in regeneration of rat liver (26). Methylation (1, 12) and phosphorylation (14, 17) also appear to alter the binding of histones to DNA and to affect both chromatin structure and function. Thus, such modifications of histone structure by group substitution reactions may change the interaction of histones not only with DNA but with the silver ions in the ammoniacal silver stain.

The observed data do not exclude either interpretation, but suggest that the ASR does detect reactive sites in either newly synthesized or in rearranged preformed arginine-rich histones.

The method appears to detect nonhistone arginine-rich proteins as well. Observations on granulocytes in chick bone marrow show a fine particulate ASR deposit in the larger secretory granules of the heterophil. The presence of an arginine-rich protein in granules of the rabbit neutrophil has been reported from several types of evidence (3, 29, 32). It seems not unlikely that chick leukocyte granules may also contain a similar arginine-rich protein; other sources of arginine-rich proteins must be investigated further.

The ASR product appears confined to the nucleus and cytoplasmic secretory granules, in which arginine-rich proteins are likely or known to occur. Mitochondria, endoplasmic reticulum, cell membranes, pancreatic zymogen granules, and extracellular spaces are all devoid of the ASR product. However, a very fine particulate deposit of the ASR is visible in the cytoplasm of reticulocytes and mature erythrocytes.

The decreased density of the cytoplasm due to a dispersal of ribosomal clusters in the hemoglobin-accumulating cytoplasm allows for the visualization of the ASR particles in their association with polysomes. These particles may be either products of interaction of silver with arginine-rich cytoplasmic proteins or merely contaminants of the method. Since extracellular space and mitochondria are consistently devoid of ASR particles, the presence of a cytoplasmic basic protein similar or identical to histones must be considered. The ASR particles are often, though not always, associated with discernible ribosomes in the immediate area of the individual particles, and may be interpreted as due to silver ion interaction with a basic protein present in ribosomes (8). Arginine in about the same total proportion as in histones has been reported in rabbit reticulocyte ribosomes (6). The possibility remains that the cytoplasmic ASR particles are indicative of silver reaction with newly synthesized arginine-rich histones on the ribosomes. Cytoplasmic synthesis of histones has been reported in HeLa cells (27), and continuing synthesis of histones in the absence of DNA synthesis has been noted in maturing erythroid cells (10), particularly in the arginine-rich fractions III and IV (12).

The ASR particle size is variable. However, the average size tends to increase with an increasing amount of deposit. The average particle size in erythrocyte nuclei appears larger than that in basophilic erythroblast nuclei. The larger size is probably due to some ionic attraction and coalescence of smaller silver particles with one another to form aggregates. When the relative amount of arginine-rich protein is low, the particle size may remain small, as in the reticulocyte cytoplasm and in heterophil secretory granules.

If a valid assumption is that the ASR is detecting arginine-rich histones in the nuclei, one may conclude that the ASR product for arginine-rich histones is first detectable during the erythroblast stage and increases in amount up to about the late polychromatophilic erythrocyte or reticulocyte stages.

These data do not add further evidence for histone as a repressor of gene action on DNA-dependent RNA synthesis. However, as indicated by the ASR deposit, arginine-rich histones, fractions III and IV, or fraction V, or both, either are formed and accumulated in the maturing stages or preexisting histones are so changed in their structure as to become reactive to AS. The reactivity coincides in time with the decrease of RNA synthesis, based on evidence of decreased uptake of radioactively labeled uridine into RNA

in duck erythrocytes (28). The appearance or increased reactivity of the histone also coincides with the increased amount of heterochromatin which is known to be inactive in RNA synthesis (4, 9, 11, 15, 19). These data are suggestive that histone changes parallel gene inactivation in erythropoiesis of the chick.

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