THE DISTRIBUTION OF NUCLEIC ACIDS IN RAT EGGS IN FERTILIZATION AND EARLY SEGMENTATION

II. HISTOCHEMICAL STUDIES

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Summary

Sections of rat eggs in various stages of fertilization and early cleavage were stained with buffered solutions of methylene blue or light green to show the distribution of basophilia and acidophilia respectively, or were treated by the Feulgen technique to show the distribution of desoxyribonucleic acid (DNA).

During fertilization the cytoplasm of the egg was evenly stained and showed weak basophilia and strong acidophilia. In the course of the first four cleavage divisions the basophilia decreased in the peripheral cytoplasm and increased in the perinuclear zone; the acidophilia retained its intensity, although in 8-cell and 16-cell eggs it was confined to the perinuclear zone.

The nucleoli of pronuclei and cleavage nuclei were very weakly basophilic and strongly acidophilic. In 4-cell, 8-cell, and 16-cell eggs a perinucleolar structure showing both intense basophilia and intense acidophilia was visible.

DNA was in general distributed as a shell about the nucleolus and as granules scattered through the nucleoplasm. In the full-grown oocyte high concentrations were evidently present in these locations. During fertilization a small amount of Feulgen-positive material was found in the early pronuclei, but none was seen in those of later stages. The concentration of DNA about the nucleoli increased greatly during cleavage, its distribution being similar to, but rather less extensive than, the perinucleolar structure just mentioned.

The results are discussed, particularly in relation to the histological findings of earlier workers and to the observations made by ultraviolet microscopy, which were described in a prior communication.

I. INTRODUCTION

In the first paper of this series (Austin and Braden 1953) an account was given of the distribution of nucleic acids in living rat eggs as determined by the absorption of ultraviolet radiation at 260 mμ. The method provided a reasonably reliable identification of nucleic acids, but could not be used to differentiate between ribonucleic acid (RNA) and desoxyribonucleic acid (DNA). To make such a distinction, it is necessary to apply histochemical procedures to sections of fixed eggs. This has been done and the results are described in the present communication.

Although many histological studies have been made on the mammalian egg during oogenesis, fertilization, and cleavage, in few have the more specific

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histochemical methods been employed. Gothie and Tsatsaris (1939) and Moricard (1949) reported observations on the distribution of DNA in the mouse oocyte, as shown by the Feulgen reaction. They found that the germinal vesicle of the full-grown oocyte did not stain, but in the oocyte just before meiosis the nucleolar membrane and the ring tetrads were Feulgen-positive. Alfert (1950) made quantitative estimates of the relative DNA contents of mouse egg nuclei. He found that the amount of DNA in the primary oocyte nucleus remains constant, so that its concentration falls as the nucleus increases in size during the growth of the oocyte. Each pronucleus in the egg during fertilization contains, according to Alfert, one-fourth the amount of DNA that is present in the primary oocyte nucleus, and each cleavage nucleus contains twice the amount of a pronucleus.

Available evidence on the general staining reactions of the nucleoli, particularly the pronuclear nucleoli, is conflicting. Earlier workers, such as Sobotta (1895), Rubaschkin (1905), Lams and Doorme (1908), Sobotta and Burckhard (1910), van der Stricht (1910), Lams (1913), and Mainland (1930), reported the presence of basophilic, non-staining, and eosinophilic nucleoli in pronuclei, the last two varieties predominating in later pronuclear development. Some nucleoli were described as having a strongly basophilic outer shell surrounding an acidophilic or non-staining interior. More recently Odor and Blandau (1951) observed uniform basophilic staining of nucleoli in early pronuclei, whereas many of the nucleoli in later pronuclei showed the basophilic shell just mentioned. In contrast to all these reports, Alfert (1950) saw no stainable nucleoli until the 4-cell and later cleavage stages, and stated: "Pronuclei and two-cell stage nuclei . . . . possess numerous Feulgen-positive shells which appear to be empty when judged by acid and basic staining." In a brief note, Flax (1951) described essentially the same finding from his investigations.

The results set forth in the present paper show a distribution of nucleic acids similar to that seen in living eggs by ultraviolet microscopy. In the pronuclei and cleavage nuclei the distribution of DNA was found to resemble that of the total nucleic acids. When treated under controlled conditions the nucleoli were always uniformly stainable, being faintly basophilic and strongly acidophilic. However, the staining reactions reported by the earlier workers could be reproduced by using the older histological methods.

II. Methods

Adult female rats, killed at the appropriate times after mating, provided full-grown oocytes, and eggs in various stages of fertilization and early cleavage. The ovaries, fallopian tubes, and uteri were removed from the animals and fixed for 18-24 hr in absolute alcohol containing 10 per cent. of glacial acetic acid. For the preservation of cell structures, and particularly the nucleoli, this fixative was found to be preferable to 10 per cent. formalin in saturated corrosive sublimate, Zenker-formol, and Flemming's weak osmic acid mixture.

Serial 8-μ sections of the paraffin-embedded tissues were stained either by the Feulgen technique as described by Stowell (1945) or by means of acid

and basic dyes, over the pH range 4-8, according to the method of Dempsey, Wislocki, and Singer (1946). Methylene blue was used as the basic dye and light green as the acid dye. The dyes were employed at a concentration of 1×10^{-4}M in phosphate or sodium hydroxide-phthalate buffers of ionic concentration 0.05. After removal of the paraffin, the sections containing eggs were left in the dye solutions at 35°C for 20 hr, after which they were washed for 5 min in water and mounted in Apathy's mounting medium. The intensity of staining was estimated visually.

Sections of eggs were also stained with Mayer's haemalum and eosin. In some instances eggs were first stained by the method of Dempsey, Wislocki, and Singer (1946), inspected, washed with alcohol to remove the dye, and then stained with haemalum and eosin. By this means differences in the staining effected by the two methods could be observed in the same structures.

TABLE 1

BASOPHILIA AND ACIDOPHILIA OF RAT EGGS DURING FERTILIZATION AND EARLY CLEAVAGE

− to ++++ = Negative to intense staining

pH	Methylene Blue					Light Green					
	Nucleoli		Perinucleolar Material (8-16-cell stage)	Cytoplasm		Nucleoli			Perinucleolar Material (8-16-cell stage)	Cytoplasm	
	Pronuclear Stages	8-16-Cell Stages		Pronuclear Stages	8-16-Cell Stages*	Pronuclear Stages	8-16-Cell Stages	Uterine Muscle Fibres		Pronuclear Stages	8-16-Cell Stages*
4	−	−	−	−	−	+++	+++	+++	++++	+++	+++
5	−	±	+	−	±	++	++	++	++	++	++
6	±	±	++	±	+	+	+	+	+	±	±
7	±	+	++++	+	++	±	±	±	±	−	−
8	±	+	++++	+	++						

* Perinuclear cytoplasm only. The rest of the cytoplasm in these stages barely stained at all.

III. OBSERVATIONS

(*a*) *Staining with Acid and Basic Dyes*

Eggs during fertilization and early cleavage were stained with methylene blue or light green over the pH range 4-8; the results are set out in Table 1. Nucleoli of pronuclei during late primary, single nucleolus, and early and late secondary growth stages (Austin 1952) were found to be similar in their reactions; they were very weakly basophilic but showed evidence of a uniformly strong acidophilia (Plate 3, Fig. 13). The nucleoli of 4-, 8-, and 16-cell eggs also exhibited strong acidophilia (Plate 3, Figs. 14 and 15) and weak basophilia (Plate 3, Figs. 16-18). The basophilia appeared a little stronger than in

the pronuclear nucleoli, but this increase was probably due to the shell of strongly basophilic material about the nucleoli of the cleaved eggs. The affinity for light green of the nucleoli in uterine muscle fibres in the sections containing 8- to 16-cell eggs was also noted in order to provide a comparison between early embryonic and somatic nucleoli. The two types of nucleoli were found to have closely similar reactions (Table 1). In eggs soon after sperm penetration, the metamorphosing sperm head and the nucleoplasm of the very early pronuclei were moderately basophilic.

Eggs stained with haematoxylin and eosin had some nucleoli which took up the haematoxylin strongly (Plate 3, Figs. 19 and 21), others which exhibited only a peripheral ring of stained material (Plate 3, Figs. 20 and 21), and others again which stained weakly with eosin. All types were seen in pronuclei during both primary and secondary growth phases. There was no obvious correlation between the size of the nucleolus and its staining reaction.

The pronuclei seen in Plate 3, Figures 19-21, are of particular interest. They had previously been stained with methylene blue by the method of Dempsey, Wislocki, and Singer (1946), when all nucleoli showed the faint uniform basophilia already referred to. The sections were then washed free of dye and stained with haemalum and eosin, and the result illustrated was obtained.

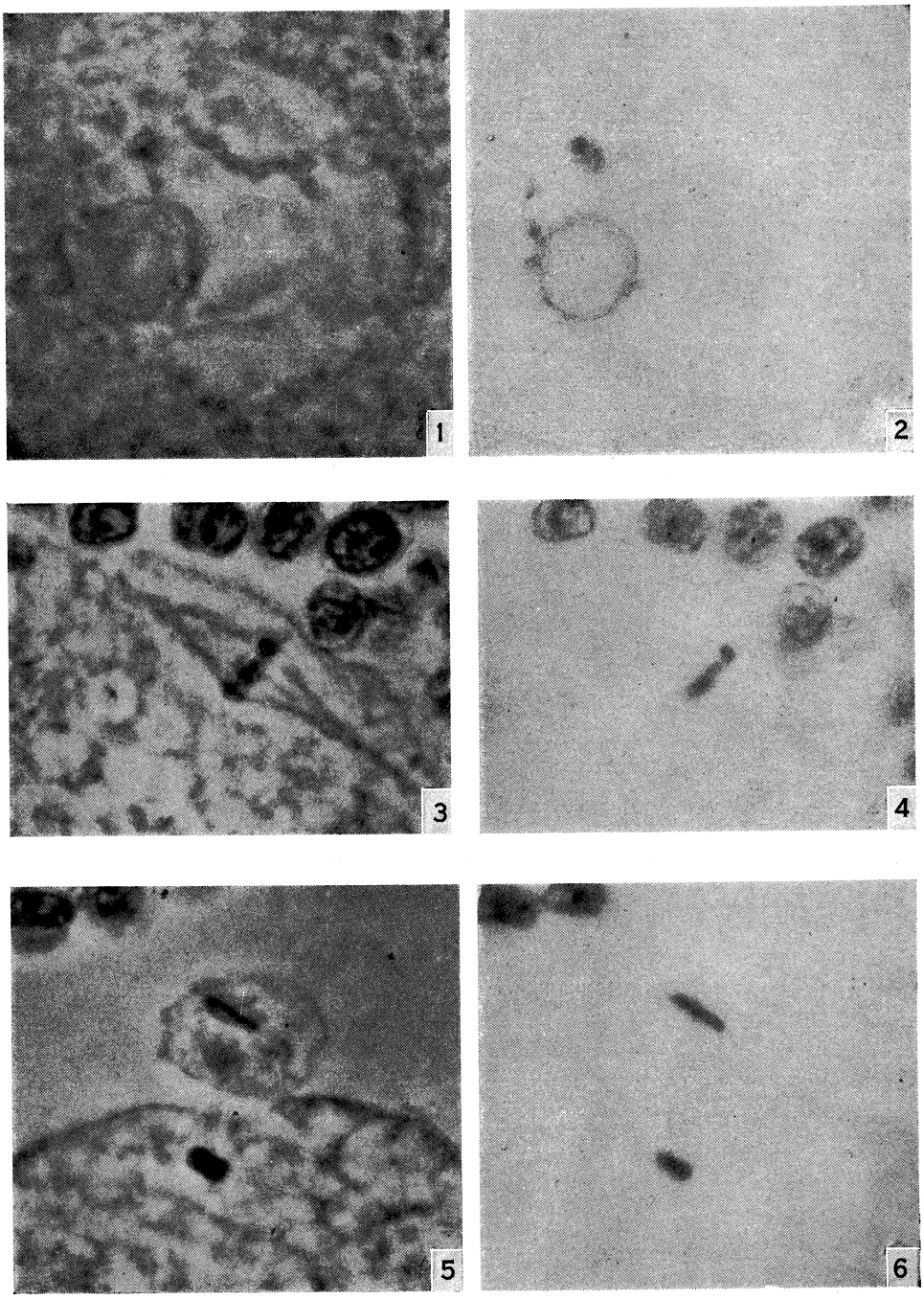
Aggregations of strongly basophilic material were regularly seen about the nucleoli in 8- to 16-cell eggs (Plate 3, Figs. 16-18) and, though less prominently, in 4-cell eggs. The perinucleolar material was also strongly acidophilic, slightly more so than the nucleoli (Plate 3, Fig. 15). The arrangement of the material was irregular but often resembled that of the perinucleolar material seen in living eggs by phase-contrast and ultraviolet microscopy (Austin 1953).

The cytoplasm of eggs in the fertilization stages was uniformly and weakly basophilic. In the early cleavage stages the basophilia was a little stronger, but was not evenly distributed; it was much more evident in the perinuclear zone than in the peripheral cytoplasm (Plate 3, Figs. 17 and 18). In the perinuclear zone the basophilia appeared to be associated with the cytoplasmic granules which were also gathered in this region. The egg cytoplasm in the fertilization stages stained evenly and strongly with light green (Plate 3, Fig. 13); in the cleavage stages the acidophilia was distributed, like the basophilia, almost exclusively in the perinuclear zone (Plate 3, Fig. 14).

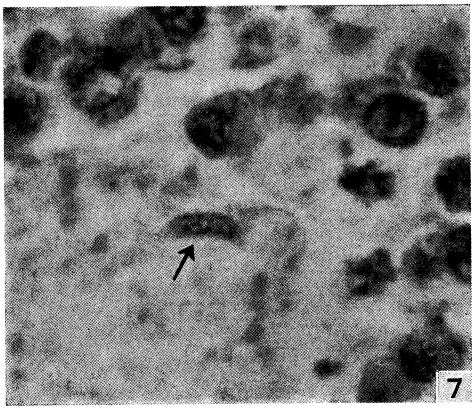
(*b*) *Staining by the Feulgen Method*

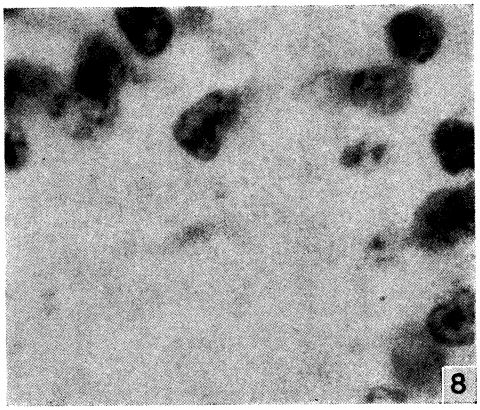
The full-grown oocyte, just prior to the first maturation division, exhibited a ring of Feulgen-positive material around the nucleolus, and some scattered granules in the rest of the nucleus (Plate 1, Fig. 2). In the unfertilized tubal egg the only stainable structure was the metaphase chromosome group of the second maturation division (Plate 1, Fig. 4). The telophase chromosomes (Plate 1, Fig. 6), the sperm head undergoing metamorphosis (Plate 2, Fig. 8), and the recently formed male and female pronuclei were all stained by the Feulgen method. As the pronuclei increased in size the staining decreased in

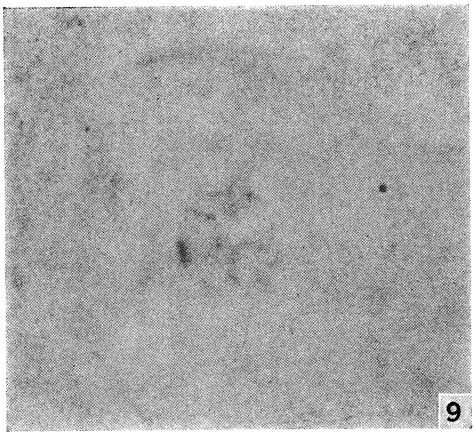
DISTRIBUTION OF NUCLEIC ACIDS IN RAT EGGS. II

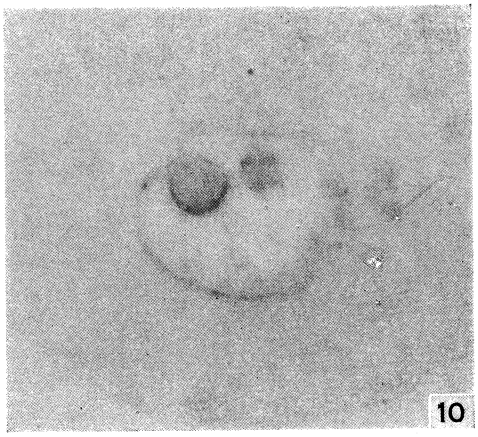


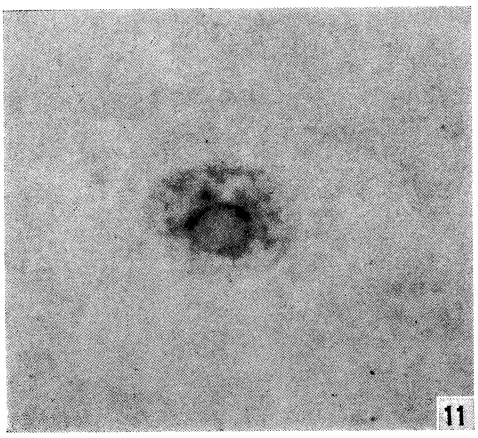
DISTRIBUTION OF NUCLEIC ACIDS IN RAT EGGS. II

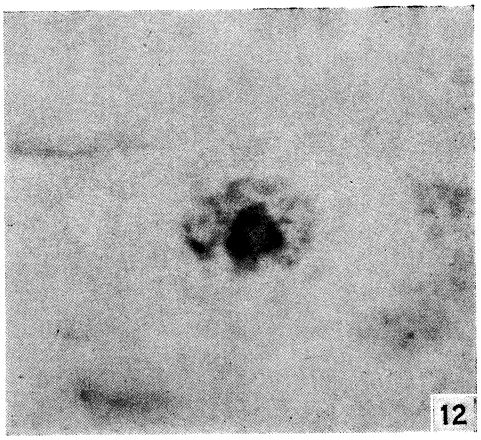












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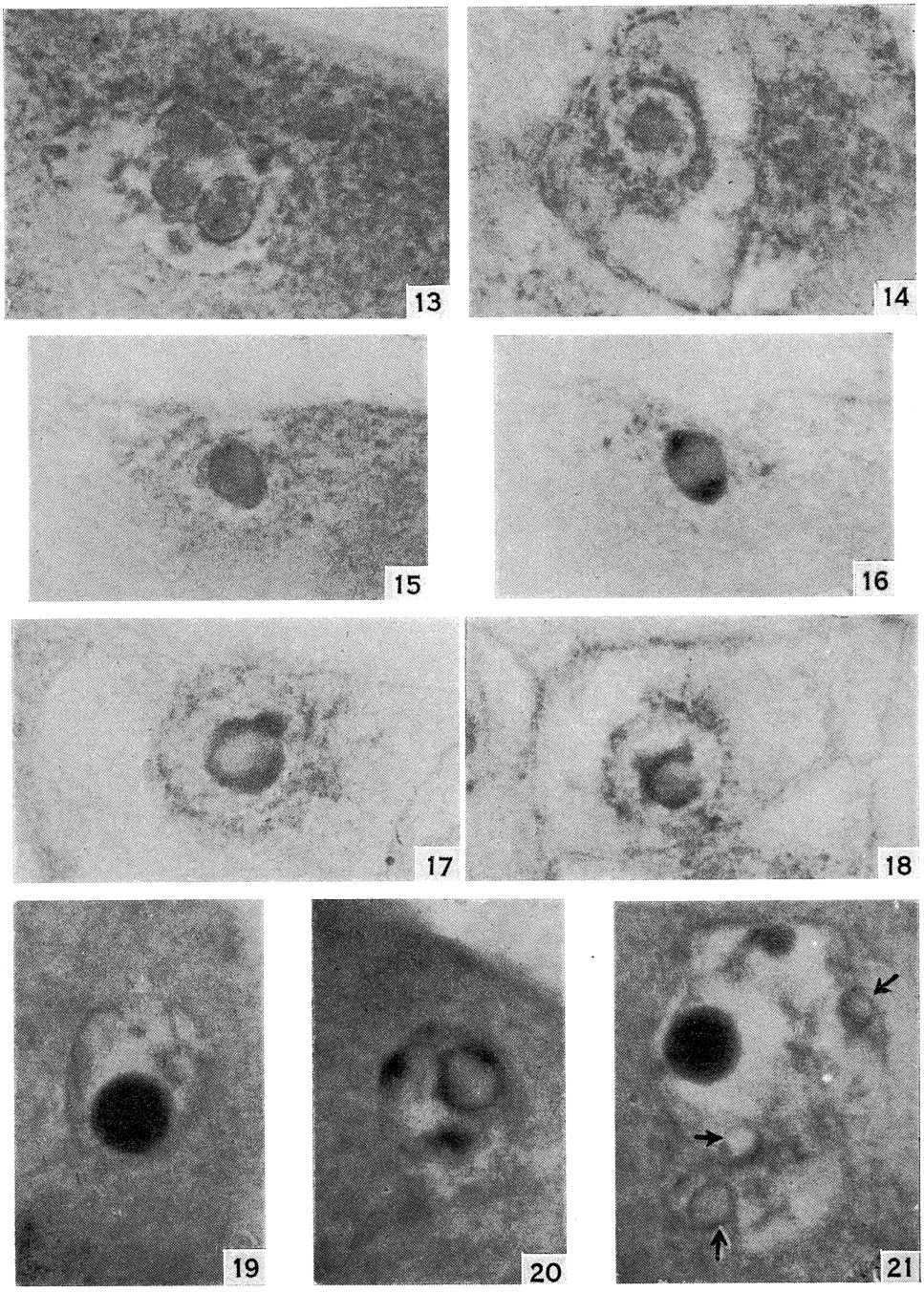

intensity (Plate 2, Fig. 9) so that at the single nucleolus stage it was barely perceptible; in the later stages of pronuclear growth no staining could be detected.

After the first cleavage division the nuclei were again Feulgen-positive. The reaction was mainly evident around the nucleoli, though there were scattered stained granules throughout the nucleus (Plate 2, Fig. 10). The distribution of Feulgen-positive material was similar in the 4-, 8-, and 16-cell nuclei, but showed a progressive increase in amount with each stage of cleavage (Plate 2, Figs. 11 and 12). In the 8- and 16-cell eggs, the stainable material around the nucleoli was irregularly disposed and often appeared to correspond to the perinucleolar basophilic structure described in the preceding section, although it was rather less voluminous.

IV. Discussion

Results described in this paper show that, when stained under controlled conditions, the nucleoli of the rat egg during fertilization and early cleavage are uniformly very weakly basophilic and strongly acidophilic. These findings differ widely from those of earlier workers, who reported a variety of staining reactions in the nucleoli, some of which were said to be non-staining, others to be strongly basophilic, and others again to be composed of a pale central region with a strongly basophilic periphery. Results of this kind were obtained also in the present investigations when the older staining methods were employed. Indeed it was possible, by staining first with methylene blue under controlled conditions and then, after removal of the dye, staining with Mayer's haemalum, to reproduce both classes of results in the same nucleoli. From these observations it is concluded that the older staining methods, involving in most instances mordanting and differentiation, and with little control of pH or ionic concentration, are unreliable for the demonstration of basophilia and acidophilia.

It is clear from the work of Pischinger (1926, 1927), Dempsey, Wislocki, and Singer (1946), Singer and Morrison (1948), and many others (see Singer 1952 for review) that a much more precise chemical characterization of tissue proteins is obtained through a study of their affinity for acid and basic dyes under controlled conditions and over a range of pH. Proteins contain both acidic and basic groups, which, by their dissociation, give rise to positive and negative charges on the molecule. As the pH is lowered the basic groups are progressively dissociated and take up more and more of the acid dye, until dye is bound to all free basic groups. Similarly, as the pH is raised more of the basic dye is taken up by the acidic groups. Apart from the free basic and acidic groups in the protein molecule itself, charged groups are often present on substances conjugated with protein. The most important of these are nucleic acids and acid polysaccharides. It is concluded therefore that the nucleoli of the rat egg during fertilization and early cleavage contain very little nucleoprotein or acid mucoprotein, and that the protein present is predominantly basic in character. Nucleoli of mammalian nerve cells (Hyden 1943) and liver cells

(Lagerstedt 1949) are also made up in part of basic proteins, but in addition they contain ribonucleic acid and hence exhibit definite basophilia as well as acidophilia.

The cytoplasm of the egg, which also exhibits weak basophilia and strong acidophilia, is presumably similar in composition to the nucleoli, in that it has much basic protein and only a low concentration of nucleic acid. The increased basophilia in the perinuclear zone in 4- to 16-cell eggs may be simply the result of an aggregation of cytoplasmic nucleic acid, previously distributed evenly through the cytoplasm. Support for the present findings lies in the fact that the observed distribution and intensity of the basophilia correspond to the distribution and concentration of nucleic acids observed in living eggs by ultraviolet microscopy and described in the first paper of this series.

A positive Feulgen reaction, connoting the presence of desoxyribonucleic acid (DNA), was particularly evident immediately around the nucleoli of the full-grown oocyte and cleavage nuclei, in the sperm head during metamorphosis, in the chromosome groups of the second polar spindle, and in the nucleoplasm of the early pronuclei. This distribution corresponds closely to the regions of strongest ultraviolet absorption noted in living eggs. In resting nuclei DNA commonly shows a perinucleolar concentration, as may be seen from the descriptions of nerve cells (Hyden 1943), erythroblasts (Thorell 1947), mammary carcinoma cells and pollen grains (Koller 1947), rat liver cells (Lagerstedt 1949), and mouse eggs (Alfert 1950). The diminution of detectable DNA in the later pronuclei may well be due simply to the dilution of the nucleic acid, present in limited amount, as the pronuclei enlarge. In much the same way, during cleavage, a constant amount of DNA could be responsible for increased concentrations through its having to aggregate about progressively smaller nucleoli. These observations are therefore consistent with the findings of Alfert (1950) for the mouse egg.

It was reported by Austin (1953) and Austin and Braden (1953) that, in living 4- to 16-cell rat eggs, a perinucleolar structure, visible by phase-contrast microscopy and showing a strong absorption of radiation at 260 $m\mu$ in the ultraviolet, became progressively more evident. They concluded that the perinucleolar material was probably analogous to Caspersson's 'nucleolus-associated chromatin' (Caspersson 1950). In the present investigation a very similar structure has been found in fixed eggs and this showed a strong basophilia when stained under controlled conditions. In sections of eggs treated by the Feulgen method high concentrations of DNA could be detected immediately about the nucleolus. Although this Feulgen-positive material was arranged in such a manner that it resembled the strongly basophilic structure, it did not appear to be quite so voluminous. There is thus a likelihood that the DNA does not account for all the basophilia of the perinucleolar material, and that the balance is due to ribonucleic acid. As the structure also has a strong affinity for light green, it presumably contains in addition basic proteins; diamino proteins are described as constituents of the nucleolus-associated chromatin.

The present observations confirm and extend the investigations made in this laboratory on the cytology of fertilization and cleavage of the rat egg (Austin

1951, 1952, 1953; Austin and Braden 1953). Information has been obtained by varied methods, which have included the use of phase-contrast and ultraviolet microscopy for living eggs and the application of histochemical methods to fixed eggs. With appropriate fixation it is possible to obtain pronuclei in all major stages of development, as well as nuclei of the first four cleavage stages, showing essentially the same nucleolar number, form, and arrangement as that seen in living eggs by phase-contrast microscopy. Findings on the distribution of the nucleic acids in both living and fixed eggs have also been in good agreement.

From the observations of morphological changes during fertilization a broad picture is obtained of the development of the pronuclei. During this process the nucleoli are formed and increase in total volume and in number. Appearances suggest that the pronuclei are engaged in some important activity which may perhaps involve the formation of gene-modified "templates" to function later in the control of protein synthesis in growth. No chemical evidence for this theory has been found, however, and it seems clear from the absence of the nucleolus-associated chromatin that a synthetic activity of the kind conceived by Caspersson and his associates does not occur during fertilization. During the segmentation of the egg, both morphological and chemical data point to the development of part of the Caspersson system, for a structure analogous to the nucleolus-associated chromatin becomes increasingly apparent. The system, however, is evidently inactive during early cleavage, for the egg is neither increasing in size nor secreting. Moreover, the nucleoli contain little or no nucleic acid and the cytoplasmic nucleic acids do not show the large increase in concentration which is said to connote synthetic activity. Of interest in this connection is Alfert's (1950) report that a great increase in cytoplasmic basophilia was observable in recently implanted mouse embryos. Intense protein synthesis begins at this time and the cytoplasmic change may well signal the arousal to activity of a system previously at rest.

V. Acknowledgments

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EXPLANATION OF PLATES 1-3

To assist in the identification of the structures, some of the figures showing distribution of Feulgen-positive material (Plate 1, Figs. 2, 4, and 6; Plate 2, Fig. 8) are accompanied by photographs of the same subject taken with the phase-contrast microscope (Plate 1, Figs. 1, 3, and 5; Plate 2, Fig. 7). Magnification throughout is × 1750.

PLATE 1

Figs. 1 and 2.—A full-grown oocyte shortly before the formation of the first maturation spindle. Feulgen-positive material is seen around the nucleolus and as granules in the nucleoplasm.

Figs. 3 and 4.—The second maturation spindle in a tubal egg, showing the strongly Feulgen-positive reaction provided by the metaphase chromosome group. The intensity of the reaction is about the same as in the nearby follicle-cell nuclei.

Figs. 5 and 6.—The second polar body soon after its extrusion. A strong Feulgen reaction is evident in the two groups of chromosomes and in the nearby follicle-cell nuclei.

PLATE 2

Figs. 7 and 8.—The sperm head in the ooplasm undergoing metamorphosis into the male pronucleus. The intensity of the Feulgen staining in this object is less than that of the nearby follicle-cell nuclei and has decreased distinctly from that shown by a sperm head before entry into the egg.

Fig. 9.—The distribution of Feulgen-positive material in an early male pronucleus is seen to be chiefly about the nucleoli and in the periphery of the nucleus. As the pronuclei grow, the intensity of staining decreases so that it cannot be detected with certainty when the pronuclei approach full development.

Figs. 10, 11, and 12.—The Feulgen staining of 2-, 8-, and 16-cell nuclei, respectively. Positively stained material is mainly perinucleolar, though much is distributed in granular form in the nucleoplasm. The increasing intensity of staining with decreasing nuclear size can easily be seen.

PLATE 3

Fig. 13.—A part-grown male pronucleus, stained with light green under controlled conditions, showing strong and uniform acidophilia in the nucleoli and cytoplasm.

Fig. 14.—A blastomere of an 8-cell egg, stained with light green under controlled conditions. Strong acidophilia is apparent in the nucleolus and in the perinuclear zone of the cytoplasm.

Figs. 15 and 16.—A nucleolus of an 8-cell egg which was stained first with methylene blue (Fig. 16), and then decolourized and stained with light green (Fig. 15), the staining being done under controlled conditions. The nucleolus itself shows faint basophilia and strong acidophilia, whereas the material attached to the nucleolus is both strongly basophilic and strongly acidophilic.

Figs. 17 and 18.—Blastomeres of 8-cell eggs stained with methylene blue under controlled conditions. Basophilia is most evident immediately about the nuclei and in the perinucleolar material.

Figs. 19-21.—Sections of eggs secured during pronuclear development and stained with Mayer's haemalum and eosin. Nucleoli can be seen which show the various forms of staining described by earlier workers. Two large and two small nucleoli show uniform intense staining; a large nucleolus has a strongly stained periphery and a pale centre, and there are three small nucleoli, indicated by arrows, which are stained peripherally only. These pronuclei had previously been stained with methylene blue under controlled conditions; all the nucleoli then showed only uniform faint basophilia. The sections were then decolourized and stained with haemalum and eosin.