THE DEVELOPMENT OF PRONUCLEI IN THE RAT EGG, WITH PARTICULAR REFERENCE TO QUANTITATIVE RELATIONS

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Summary

The development of pronuclei has been studied by examining rat eggs freshly recovered at various times between ovulation and the first segmentation.

Pronuclear development is divided for convenience into six stages on the basis of the changes occurring in the male pronucleus. By noting the frequency with which the stages were observed the relative duration of each stage has been estimated.

The diameters of the nucleoli and pronuclei were measured at each stage of pronuclear development. The data include the numbers of nucleoli in each pronucleus, and calculations are made of the volumes of nucleoli and pronuclei, and the total surface areas of the nucleoli.

In the male pronucleus the mean number of nucleoli shows an early rapid fall from an initial figure of 7.5 to about one, and then a rise to a level of about 17. On the other hand the mean number of nucleoli in the female pronucleus increases steadily from about three in the first stage to nine in the final stage.

The volumes of the pronuclei increase to reach a maximum in the second half of pronuclear development. The final volume of the male pronucleus lies between 5000 and 6000 cu. μ and that of the female between 2000 and 2500 cu. μ.

The total volume of the nucleoli in each pronucleus increases rapidly to achieve a level in the first third of pronuclear development which is maintained for the remaining two-thirds. In the male pronucleus the maximum total nucleolar volume was about 550 cu. μ, and in the female about 220 cu. μ.

The total surface area of the nucleoli reaches a maximum more rapidly in the female pronucleus than in the male, the difference being referable to the formation of a single nucleolus at an early stage in the male. Maximum values for the total surface area of nucleoli were about 730 sq. μ in the male pronucleus, and about 345 sq. μ in the female.

I. Introduction

The earliest detailed description of the changes shown by the pronuclei of the mammalian egg is probably to be found in the classical paper by Sobotta (1895) on the mouse egg. According to Sobotta, the early pronuclei contain chromatin in dense strands with irregular nodal thickenings; later the chromatin is formed into nucleoli. He considered that the male pronucleus always has only one large nucleolus, the female either one or, more commonly, several.

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Sobotta noted that, with further development, the nucleoli begin to stain lighter and the chromatin breaks up and becomes irregularly scattered on the achromatic network. Finally the nucleoli become clear sacs, only their outlines remaining.

Lams and Doorme (1908), who also investigated the mouse egg, noted that the early nucleoli have a transitory existence; at a certain stage the chromatin contained in the nucleoli undergoes fragmentation into little masses situated closely in contact with the nuclear membrane and along fine filaments. They observed that these filaments seem to be disposed more or less radially about a large "plasmatic nucleolus" which is always excentric in position. At a later stage the chromatic granulations were found to exist in larger numbers, but the nucleolus was always non-staining and homogeneous.

In the rat egg, Sobotta and Burckhard (1910) observed changes broadly similar to those Sobotta had described for the mouse egg: the pronuclei in the early stages contain chromatin masses and a linin network; later the nucleoli disappear and a very distinct network is present instead.

The pronuclei of the guinea pig egg were found by Lams (1913) to contain a fine reticulum, between the threads of which were granules and chromatic globules, rodlets, and "nucleinic" nucleoli. According to this author the nucleoli initially are clearly "nucleinic"; towards the time when the chromosomes of the first segmentation spindle become visible the nucleoli are "plasmatic."

Kremer (1924) re-examined Sobotta's preparations of mouse and rat eggs and attempted a logical account of the growth and development of pronuclei. He maintained that pronuclear growth could be divided into two stages: a stage of chromatin increase, and a stage of chromatin reduction. In the first stage the early pronuclei contain small chromatic masses or globules of varying size, some of which take the iron haematoxylin stain more weakly than others and some show outer dark and inner clear zones. Kremer believed that the globules grow and coalesce, drawing material from the cytoplasm, until a single large nucleolus, the "principal nucleolus," is formed. The second stage, which he described, is characterized by a reduction in the accumulated chromatic substance: the "principal nucleolus" loses its staining affinity, the chromatin is split up and, in the form of fragments, is passed back into the cytoplasm. Kremer observed chromatic bodies, resembling small nucleoli, lying scattered through the cytoplasm and considered these to be the nucleolar fragments that had migrated from the pronucleus.

Kremer's theory was that the pronuclear bodies are synthesized from ooplasmic material and, in the pronuclei, are brought into close chemical relations with the specific maternal and paternal nuclear constituents. After the nuclear chromatin has reached its greatest accumulation the now adequately treated substances leave the pronuclei in the form of chromatic fragments. Kremer suggested that each of these may be regarded as a carrier of maternal or paternal hereditary qualities, which are thus distributed over the whole egg and pass equally into all descendant cells.

Mainland (1930) considered that the pronuclei of the ferret egg pass from a condition of uniformly stained chromatic material to one in which this

material is in the form of globules and granules of various sizes, the larger and medium-sized particles occurring at later periods. Occasionally he saw, even in an early pronucleus, a more or less spherical body with a darkly stained periphery and a pale centre. In general, eosinophilic, pale, or colourless particles were much more commonly found in the later central than in the earlier peripheral pronuclei. Mainland could find no evidence of a removal of chromatin from the pronuclei such as Kremer claimed.

In the mouse egg, Gresson (1942) noted that the male pronucleus early contains a single nucleolus but with the rapid increase in size of the pronucleus several nucleoli appear. The female pronucleus, on the other hand, had several nucleoli, sometimes as many as 10 or 11. In later states, according to Gresson, the pronuclei are about the same size and have the same number of nucleoli.

All these investigators made their observations on histological material and it is apparent that a concept of pronuclear development based upon their descriptions is both incomplete and confusing. A proper understanding of the nature and significance of the fertilization process requires an accurate knowledge of pronuclear development, but it seems unlikely that this can be obtained from material prepared by the older histological methods. A new approach to the problem has recently been attempted by examining living eggs with the phase-contrast microscope (Austin 1951). Observations described in that paper were made chiefly on the process of fertilization as it took place *in vitro*. The present paper concerns the study of freshly recovered eggs, which were examined with the object of determining the form of the changes occurring *in vivo* and of obtaining quantitative data on the development of the pronuclei.

II. Methods and Materials

The materials and most of the procedures employed were similar to those described in a previous communication (Austin 1951). In the present investigations, however, eggs were recovered from the fallopian tubes in saline and not under paraffin as they were not required for protracted observation.

III. Observations

(*a*) *General Course of Pronuclear Development*

On the basis of experience previously described (Austin 1951), and that obtained subsequently, it has been found convenient to consider the process of pronuclear development as passing through six consecutive stages. These have been named, respectively, early and late primary growth, single nucleolus stage, early and late secondary growth, and, finally, pronucleus at full development. These stages are based upon the changes shown by the male pronucleus, which are much more distinct than those occurring in the female pronucleus.

Early primary growth begins with the first appearance of "primary" nucleoli in the structure representing the metamorphosed sperm head, and includes the initial enlargement of these nucleoli (Plate 1, Fig. 1). The nucleoli

show further growth during the late primary growth stage (Plate 1, Fig. 2) and also undergo coalescence so that generally a single large primary nucleolus is formed.

For a period the large primary nucleolus continues to enlarge at a rate seemingly parallel to that at which the pronucleus enlarges, but later the pronucleus begins to grow more quickly. This is referred to as the single nucleolus stage (Plate 1, Fig. 3).

In the early secondary growth stage (Plate 1, Fig. 4) the rate of growth of the pronucleus clearly exceeds that of the primary nucleolus and more free space becomes apparent within the pronucleus. Soon new nucleoli make their appearance at the periphery of the pronucleus; these are called the "secondary" nucleoli. As they grow the secondary nucleoli begin to move away from the periphery.

The stage of late secondary growth is characterized by the still larger size of the pronucleus and by the presence of a number of secondary nucleoli distributed both on the nuclear membrane and nearer the centre of the pronucleus (Plate 1, Fig. 5). The large primary nucleolus undergoes a distinct reduction in size during this stage.

In the pronuclei that have reached the stage of full development there is a larger number of secondary nucleoli and many of these have acquired a moderate size (Plate 1, Fig. 6). The single large primary nucleolus has become still smaller so that it is now not much larger than the secondary nucleoli. At this stage the nucleoli are grouped in the central region of the pronucleus, away from the nuclear membrane.

The development of the female pronucleus differs from that of the male. The nucleoli make their appearance from the chromosome group at the vitelline end of the second maturation spindle. As they grow the nucleoli undergo little coalescence so that rarely is a single nucleolus formed. Secondary nucleoli begin to appear earlier than in the male pronucleus but the total number of nucleoli ultimately found is only about half that of the male pronucleus.

In the male pronucleus the nucleoli lie, in the main, away from the nuclear membrane, except during early and late secondary growth, particularly the former, when secondary nucleoli are found on the nuclear membrane and may even be slightly flattened against it. The nucleoli of the female pronucleus, on the other hand, are mostly to be seen near the nuclear membrane throughout development. In the stages of growth that correspond to late primary growth, single nucleolus, and early secondary growth of the male pronucleus the nucleoli in the female pronucleus are often found deeply embedded in the nuclear membrane (Plate 1, Fig. 7). For the whole period of development the male pronucleus is larger than the female.

The stage of full development is terminated by the rapid reduction of the pronuclei and nucleoli, the disappearance of the nuclear membrane, and finally the dissolution of the nucleoli to give place to the prophase chromosomes of the first segmentation mitosis. The details of these changes as they were

seen to occur *in vitro* have been described (Austin 1951). Examination of freshly recovered eggs at the appropriate stages has indicated that the process is essentially the same *in vivo*.

(*b*) *Relative Duration of Stages in Pronuclear Development*

To obtain a measure of the relative duration of the developmental stages described in the last section, mature female rats were placed with males in the evening and examined the following morning for evidence of mating. Rats with copulation plugs were killed at 6-hourly intervals from 8.0 a.m. on the first day to 8.0 a.m. on the following day, and the eggs were examined to determine the stage of fertilization. Data were obtained from five rats at each of the times indicated (Table 1).

TABLE 1

NUMBER OF EGGS IN DIFFERENT STAGES OF FERTILIZATION WHEN FRESHLY RECOVERED FROM RATS KILLED AT VARIOUS TIMES DURING THE DAY AND NIGHT FOLLOWING MATING

Hour	Number of Rats Used	Number of Unpenetrated Eggs	Number of Eggs in Pre-Pronuclear Stages	Number of Eggs in Pronuclear Stages					Number of Eggs in Post-Pronuclear Stages	Total Eggs
				Primary Growth	Single Nucleolus	Early Secondary Growth	Late Secondary Growth	Full Development		
8.0 a.m.	5	3	42	6	1	0	0	0	0	52
2.0 p.m.	5	0	0	12	10	17	11	0	0	50
8.0 p.m.	5	3	1	0	0	11	12	19	0	46
2.0 a.m.	5	1	0	0	0	0	2	30	12	45
8.0 a.m.	5	1	0	0	0	0	0	7	41	49
Totals	25	8	43	18	11	28	25	56	53	242

Most (45 out of 52) of the eggs obtained from the rats killed at 8.0 a.m. on the first day either had no sperm within (three eggs) or were in the stages of fertilization preceding pronucleus formation (42 eggs). There were, however, six eggs with pronuclei in primary growth, and one in the single nucleolus stage. Rats killed at 2.0 p.m. yielded 50 eggs, all with pronuclei; in 11 of these the pronuclei had reached the stage of late secondary growth. At 8.0 p.m., three eggs were unpenetrated and one had a sperm in the perivitelline space, the remaining 42 eggs having pronuclei in the stages of early and late secondary growth, and full development, principally the last-named. At 2.0 a.m. the following morning most of the eggs (30 out of 45) were in the stage of full development and 12 were either in the segmentation stages or had already divided; one egg was unpenetrated.

In order to obtain from these data the approximate duration of each of the pronuclear stages, two methods may be used. By the first method the relative duration is assumed to be proportional to the frequency with which each stage is observed. This is the more direct method but is open to the objection that the rats were killed at set intervals and not at random times,

thus possibly favouring certain pronuclear stages. However, the results so obtained are essentially the same as those that can be derived by the use of the second method, the calculation of the cumulative percentages of eggs passing through each stage. Because of its simplicity the former method is followed here.

The total number of eggs having pronuclei in each of the stages of primary growth, single nucleolus, early and late secondary growth, and full development were 18, 11, 28, 25, and 56 respectively. It can be seen that the single nucleolus stage is the shortest, that early and late secondary growth are each about two and a half times as long and full development about five times as long. Primary growth is subdivided into early and late phases of approximately equal duration, each of which would be a little shorter than the single nucleolus stage. This information is used below for the expression of the approximate rates of dimensional change in pronuclei and nucleoli during the course of pronuclear development.

(*c*) *Quantitative Changes in Pronuclear Development*

Adult female rats were placed with males in the evening and examined the following morning. Those with copulation plugs were killed at times varying from 8.0 a.m. the same day to 8.0 a.m. the following day. In this way eggs were obtained with pronuclei in all the stages described in Section (*a*) above.

The eggs recovered were classified according to the stage of pronuclear development. The diameter of each nucleolus, and the longest and shortest horizontal diameters of the pronuclei, were measured with an eyepiece micrometer. The vertical depth of each pronucleus was estimated with the aid of the calibrated fine adjustment on the microscope.

Altogether 96 eggs were examined in this way and the following information was obtained:

(i) *Number of Nucleoli* (Table 3).—The number of nucleoli in the female pronucleus showed a steady increase from a mean of three in the early primary growth stage to a mean of about nine at full development (Fig. 1*A*). In this series a single nucleolus was not seen in the female pronucleus, but in two eggs there were only two nucleoli in each of the female pronuclei. The male pronuclei of these eggs were at the single nucleolus stage.

The mean number of nucleoli in the male pronuclei, initially between seven and eight, showed a steep fall to the single nucleolus stage. In only one egg out of 14 at this stage was there more than one primary nucleolus seen and this egg had two. During the secondary growth phase the mean number of nucleoli rapidly increased to reach a maximum of about 17 (Fig. 1*A*).

(ii) *Volumes of Pronuclei and Nucleoli* (Table 2).—The volumes of both male and female pronuclei increased slowly in the primary growth phase, more rapidly during early secondary growth, and then more slowly to form a plateau at the stage of full development (Fig. 1*B*). At maximum size the mean volume

TABLE 2

TOTAL VOLUMES OF NUCLEOLI AND VOLUMES (CU. μ) OF PRONUCLEI IN EGGS EXAMINED AT VARIOUS STAGES OF PRONUCLEAR DEVELOPMENT

Stage of Pronuclear Development	No. of Eggs Examined	Male				Female			
		Nucleoli		Pronuclei		Nucleoli		Pronuclei	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean
Early primary	4	11-143	56.8 ± 29.96*	83-445	236.5 ± 90.96*	0-58	22.5 ± 13.35*	0-126	67.8 ± 29.45*
Late primary	9	135-339	228.3 ± 26.88	523-1400	842.8 ± 112.40	35-131	83.8 ± 10.53	160-513	300.4 ± 41.27
Single nucleolus	14	253-579	423.5 ± 23.13	787-1867	1261.8 ± 74.01	103-221	154.8 ± 9.64	224-865	562.9 ± 46.48
Early secondary	26	382-917	566.3 ± 23.53	1731-5824	3098.7 ± 217.62	136-377	213.2 ± 9.64	485-1817	1084.4 ± 64.52
Late secondary	26	359-721	544.8 ± 19.41	2932-6751	5269.9 ± 198.87	180-269	231.2 ± 5.32	1031-2673	2007.9 ± 97.36
Full development	17	481-662	550.9 ± 13.56	4340-6842	5702.7 ± 161.04	170-276	221.2 ± 7.94	1764-2810	2314.2 ± 66.19

* Standard error.

of the male pronuclei was about 5700 cu. μ and of the female pronuclei about 2300 cu. μ. The growth rates of the male and the female pronuclei were closely similar so that the mean volumes of the male pronuclei remained throughout at about two and a half times the mean volumes of the female pronuclei.

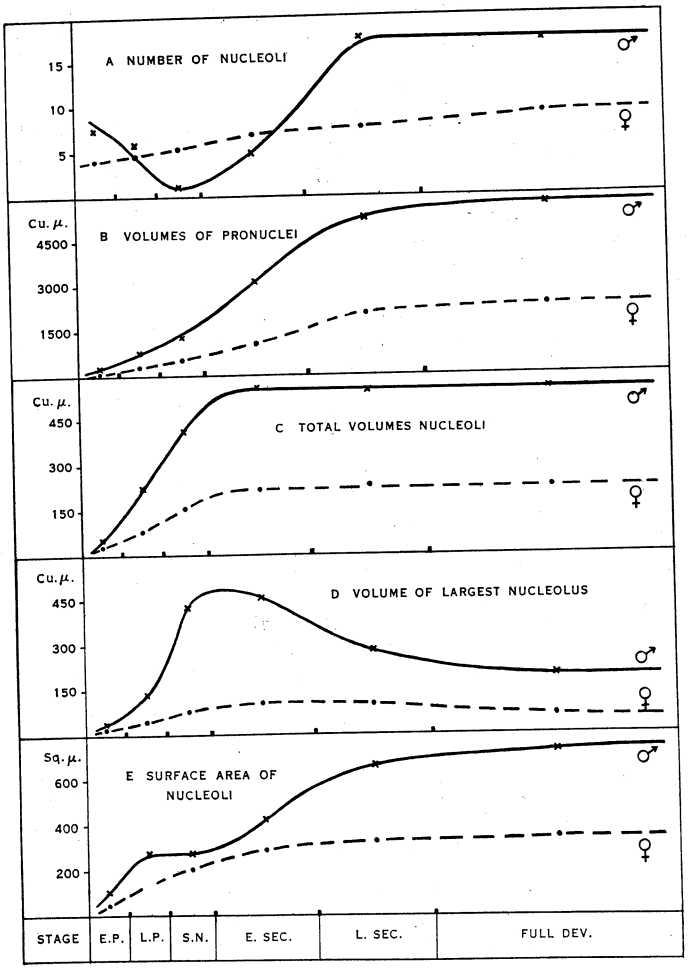


Fig. 1.—Showing the changes which take place during pronuclear development in the number, volume, and surface area of nucleoli, and in the volume of the pronuclei. Stages of pronuclear development indicated are: E.P., early primary growth; L.P., late primary growth; S.N., single nucleolus stage; E.Sec., early secondary growth; L.Sec., late secondary growth; Full Dev., full development.

The growth curves for the mean total volumes of nucleoli are quite different in form from the growth curves of the pronuclear volumes (Fig. 1C). Total nucleolar volumes increased rapidly during the primary growth and single nucleolus stages and reached a plateau in the early secondary growth stage.

TABLE 3

NUMBERS AND TOTAL SURFACE AREAS OF NUCLEOLI AND VOLUMES OF LARGEST NUCLEOLI AT VARIOUS STAGES OF PRONUCLEAR DEVELOPMENT

Stage of Pronuclear Development	No. of Eggs Examined	Nucleoli of Male Pronucleus						Nucleoli of Female Pronucleus					
		Number of Nucleoli		Total Surface Area of Nucleoli (sq. μ)		Volume of Largest Nucleolus (cu. μ)		Number of Nucleoli		Total Surface Area of Nucleoli (sq. μ)		Volume of Largest Nucleolus (cu. μ)	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Early primary	4	4-9	7.5 ± 1.19*	77-163	107.8 ± 26.80*	6-131	39.3 ± 30.60*	0-6	3.0 ± 1.29*	0-87	45.0 ± 20.01*	0-48	16.25 ± 11.20*
Late primary	9	2-12	5.9 ± 1.03	220-353	280.0 ± 15.77	35-326	136.8 ± 36.52	2-7	4.6 ± 0.64	91-201	138.6 ± 13.32	16-104	47.4 ± 9.43
Single nucleolus	14	1-2	1.07 ± 0.07	206-337	273.4 ± 10.04	253-579	423.1 ± 23.91	2-8	5.3 ± 0.55	54-291	203.4 ± 16.14	35-161	83.5 ± 10.62
Early secondary	26	1-11	4.7 ± 0.59	254-646	427.3 ± 19.09	234-839	456.1 ± 32.42	3-9	6.9 ± 0.33	201-381	287.8 ± 9.61	48-326	112.1 ± 11.60
Late secondary	26	4-30	17.5 ± 1.37	485-910	660.9 ± 21.26	48-579	280.5 ± 31.84	3-14	7.5 ± 0.47	227-433	321.8 ± 9.98	35-195	98.8 ± .8.46
Full development	17	5-36	17.1 ± 2.23	481-909	730.1 ± 30.79	82-442	203.8 ± 20.84	5-12	9.0 ± 0.49	234-390	345.2 ± 9.57	35-104	65.4 ± 6.02

* Standard error.

The volumes then remained constant throughout the stages of late secondary growth and full development. The final volume achieved by the nucleoli of the male pronuclei, about 550 cu. μ, was twice that of the nucleoli of the female pronuclei, about 225 cu. μ. During the initial phase of growth the volume of the male nucleoli increased at a greater rate than that of the female nucleoli.

The mean volume of the largest nucleolus in the male and female pronuclei was also calculated (Table 3, Fig. 1*D*). In the female pronuclei the volume of the largest nucleolus increased fairly rapidly to a maximum of about 100 cu. μ at the early secondary stage and then declined slowly to about 60 cu. μ at the stage of full development. The volume of the largest nucleolus in the male pronuclei showed a much more rapid increase during primary growth and reached a maximum of about 450 cu. μ in the early secondary growth stage. Later, particularly during late secondary growth, the volume decreased to a terminal value of about 200 cu. μ.

(iii) *Surface Area of Nucleoli* (Table 3).—The total surface areas of the nucleoli increased throughout pronuclear development and in much the same general manner in both male and female pronuclei (Fig. 1*E*). The increase was most rapid in the early stages. The final surface area achieved by the nucleoli in the male pronucleus, about 730 sq. μ, was twice as large as in the female pronucleus, about 350 sq. μ.

The curve for the nucleolar surface area in the male pronucleus showed a distinct discursion with the formation of the single nucleolus, but the increase in surface area was resumed during the stage of early secondary growth.

IV. Discussion

The observations on freshly recovered eggs described in this paper indicate that the steps involved in the early phase of pronuclear development *in vivo* are essentially the same as those observed *in vitro* and described in a previous communication (Austin 1951). The only notable difference lies in the evidence that a single nucleolus is not usually formed in the female pronucleus *in vivo*, whereas it has been observed to form in several eggs studied *in vitro*.

It seems likely that the regular formation of a single nucleolus in the male pronucleus can be ascribed to limitation of space. Contact between nucleoli generally results in coalescence, and nucleolar volume increases so rapidly during the phase of late primary growth that the pronucleus becomes incapable of accommodating two or more nucleoli without their coming in contact with one another. This applies rather more to the male pronucleus for, although the male and female pronuclei grow at about the same rate, nucleolar volume increases more rapidly in the male.

The general pattern of pronuclear development, involving the formation of a single large nucleolus from a number of small nucleoli, and its later replacement by a number of medium-sized nucleoli, is in agreement with the observations of previous workers. However, by examining living eggs it has been possible to obtain further information, including quantitative data, on the changes that take place during pronuclear development.

The total volume of the nucleoli in both pronuclei is found to increase rapidly from their first appearance until a maximum is reached in the early secondary growth stage. This level is maintained until the terminal sudden decrease in volume which occurs when the nucleoli give place to the prophase chromosomes of the first segmentation mitosis.

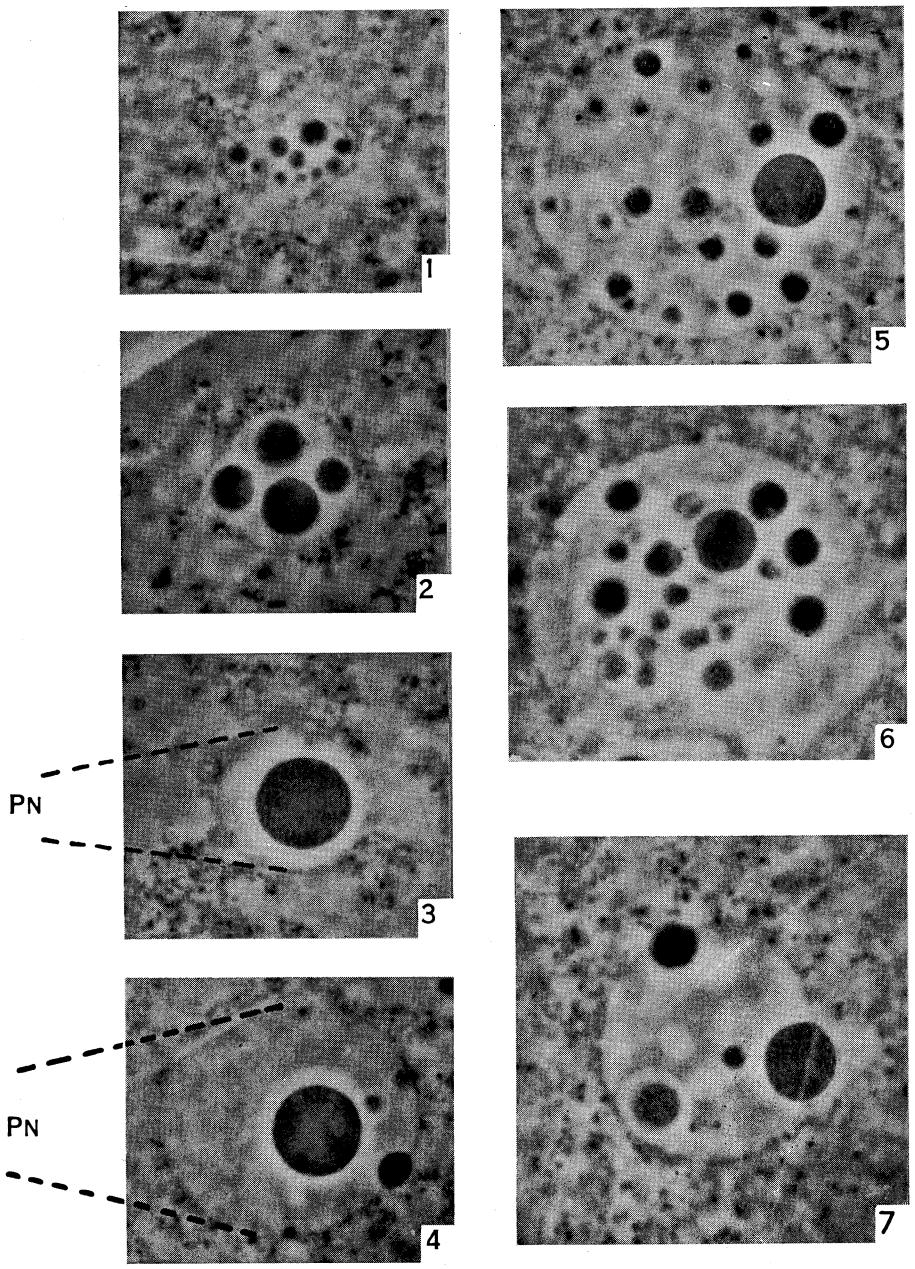
Although the volume remains constant the number of nucleoli continues to increase after the early secondary growth stage. This is accompanied in both pronuclei by a reduction in size of the largest nucleoli and the growth of the newly formed secondary nucleoli; the change is apparent in the male pronucleus much more than in the female. There is thus a redistribution of nucleolar material during the secondary growth stages. This does not appear to take place by a process of fragmentation as Kremer (1924) suggests, for if fragmentation were involved the secondary nucleoli would surely be found first in the near vicinity of the large primary nucleolus; on the contrary they regularly occur near the periphery of the pronucleus. The indications are, in fact, that the secondary nucleoli originate on the nuclear membrane. There is no evidence for the migration of nucleoli out of the nucleus and into the cytoplasm; rather the movement is towards the centre of the nucleus.

As expected from the other observations, the total surface area of the nucleoli was found to increase throughout pronuclear development even though total nucleolar volume remained constant for most of this period.

Seen through the phase-contrast microscope, the nucleoli appear as smooth, uniformly black spheres that differ from each other only in size. On the other hand, most of the previous investigators have described differences in the staining properties of the nucleoli, which suggests that there exist corresponding differences in the chemical nature of the nucleoli. It seems more likely, however, that the effects observed are referable to weakly or negatively basophilic nucleoli surrounded by a layer of strongly basophilic material which is present in limited total quantity. In these circumstances the appearance of the nucleoli in histological preparations would vary with the plane of section and with the effects of fixatives. Nucleoli with pale centre and darkly stained periphery have been described by both Kremer (1924) and Mainland (1930). In the later stages of pronuclear development a large proportion of lightly stained or unstained nucleoli would be expected, because of the increase in surface area, and such a distribution has been recorded by Mainland.

These ideas are consistent with the concept of the nucleolus and its surrounding layer of associated chromatin that has been developed by Caspersson and his associates (Caspersson 1950). It is not possible, however, to determine whether the same system exists in rat pronuclei by examining living eggs with the phase-contrast microscope. Information from more specific methods is required and it is proposed to further these investigations with the aid of the Feulgen reaction and ultraviolet microscopy.

DEVELOPMENT OF PRONUCLEI IN THE RAT EGG



V. Acknowledgment

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Explanation of Plate 1

The photographs were taken with the aid of a phase-contrast microscope. Magnification is x1400 for Figures 1-6, and x1800 for Figure 7.

Figures 1-6 illustrate the male pronucleus in the stages of pronuclear development referred to in the text.

Fig. 1.—Early primary growth.

Fig. 2.—Late primary growth.

Fig. 3.—Single nucleolus stage. Limits of pronucleus indicated (Pn).

Fig. 4.—Early secondary growth. Limits of pronucleus indicated (Pn).

Fig. 5.—Late secondary growth.

Fig. 6.—Full development.

Fig. 7.—Female pronucleus showing nucleoli embedded in the nuclear membrane.