SYNTHESIS OF YOLK IN OOCYTES OF RANA PIPIENS AFTER INDUCED OVULATION[1]

NORMAN E. KEMP
Department of Zoology, University of Michigan, Ann Arbor

FOURTEEN FIGURES

INTRODUCTION

The general sequence of deposition of yolk platelets in growing amphibian oocytes has been studied by a number of investigators, including King ('08), Jörgensen ('13), Hibbard ('28), Fischer ('32), Brachet ('50) and Wittek ('52); and the distribution of platelets in the mature primary oocyte has been described by Daniel and Yarwood ('39), Pasteels ('46) and Wittek ('52). Immediately after ovulation in an adult female of Rana pipiens, the ovaries are devoid of enlarged pigmented oocytes but possess numerous small oocytes, some of which within a few days begin to synthesize yolk and to grow. Yolk first appears at the periphery of the oocyte and accumulates centripetally until about three-fourths of the cytosome is filled with platelets. A narrow cortical zone still remains free of platelets and only a few scattered platelets are found in the basophilic perinuclear zone. Pigment now appears at the periphery, at first only in the animal hemisphere. Active feeding provides the nutrition necessary for continued synthesis of yolk, pigment and lipochondria (Holtfreter, '46) until the oocyte reaches maturity, at which time yolk platelets fill the cytosome. In nature Rana pipiens breeds in April or May and by September or October (Rugh, '37, '48; Ryan and Grant, '40) oocytes destined to be laid the following spring have

[1] Aided by grants from the Horace H. Rackham School of Graduate Studies and the Michigan Memorial-Phoenix Project, University of Michigan.

reached full size and may be caused to ovulate artificially by injection of pituitary glands.

Speculation on the mechanism of synthesis of yolk in Amphibia has gone hand in hand with accumulating cytological information. King ('08) reviews the earlier literature and credits Henneguy with being the first to theorize (in 1893) that nucleolar substances give rise to the "yolk nucleus," a cytoplasmic mass believed homologous to the nucleus of Balbiani in mammalian oocytes and considered responsible for the synthesis of yolk. She gives Will credit for the statement (in 1884) that nucleolar substance migrates from the nucleus to the periphery of the oocyte and there fragments to form platelets. Lams ('07) surmised that the yolk nuclei ("masse vitellogène") in Rana temporaria were derived from archoplasm, which includes the centrosome, because of their polarized accumulation on one side of the germinal vesicle. He considered that the yolk nuclei were actually mitochondria and that by enlargement they gave rise to the yolk platelets first appearing at the periphery. King ('08) describes the appearance of cytoplasmic "vitelline bodies" in early oocytes of Bufo lentiginosus, the enlargement of these to form "yolk nuclei," and the subsequent breakup of the latter to form yolk platelets. She was unable to decide whether the vitelline bodies were derived from nucleoli, chromatin, follicle cells or directly from ground cytoplasm. Gatenby and Woodger ('20) favor the theory of origin of yolk from the mitochondria or Golgi apparatus. Hibbard ('28) proposes that the platelets are probably of compound origin — from Golgi apparatus, ground cytoplasm and substances of nuclear origin. The interesting findings of Worley ('43, '44) on the close relationship between Golgi bodies and turnover of both albuminous and fatty yolk, studied in vitally or supravitally stained living eggs and developing embryos of molluscs, suggest the possibility that the Golgi apparatus is more important in the metabolism of deutoplasm than is commonly realized. More recently, Panijel ('50) has advanced the idea that cytoplasmic microsomes may enlarge to form visible granules which,

in turn, grow directly into yolk platelets. Wittek ('52) subscribes to the theory that nucleoli play an important role in the synthesis of cytoplasmic proteins, possibly, as Brachet has suggested, by controlling the synthesis or multiplication of microsomes, which then intervene directly in synthesis of proteins.

It can be seen from the above that the exact origin of yolk in amphibians is still an open problem. Theoretically, the use of radioactive tracer elements would be a valuable new technique for following the synthesis of yolk. Friedberg and Eakin ('49) and Eakin, Kutsky and Berg ('51) have demonstrated uptake of glycine and methionine containing C-14 in cut halves of embryos of Hyla regilla or Rana pipiens. Although there was apparently no uptake in the yolk platelets of their specimens, it is logical to anticipate that amino acids would easily be incorporated into the yolk platelets during their active synthesis in the oocyte. For the success of experiments with tracers, however, precise information is needed on the time required for oocytes to complete various stages of vitellogenesis. A search of the literature has failed to reveal any quantitative data on this subject; hence the present paper deals with an attempt to follow temporally the deposition of yolk in adult female frogs maintained in the laboratory subsequent to artificially induced ovulation.

MATERIALS AND METHODS

Large, mature females of Rana pipiens purchased from a dealer in Wisconsin and stored in a cold room at 4°C. were induced to ovulate by injecting fresh pituitary glands. A total of 108 animals were injected at various times from January to May, 1951 and 1952. Of these, 20 in the 1951 series and 11 in the 1952 series ovulated well and survived long enough to be included in an experimental series of individuals sacrificed at approximately 5- or 10-day intervals up to 120 days after ovulation. In addition to the animals maintained for definite periods after ovulation, 36 animals re-

ceived during the last weeks of July, August and September, 1952, were sacrificed without prior pituitary injection in order to compare the natural rate of growth of oocytes with that achieved in laboratory-fed animals.

Experimental animals were kept at room temperature and stripped as completely as possible one or two days after injection. Many of the earlier experimental animals died before it was recognized that it was necessary to feed females soon after stripping. Animals stored for long periods in the cold room and then brought to room temperature evidently developed severe malnutrition. Very few died after it became routine procedure to start feeding them right after stripping and daily thereafter until the time of sacrifice. Since the animals had to be force-fed, it was convenient to serve them canned dog food or a mixture of dog food and strained spinach, foods which kept well in a refrigerator operating at about 10°C. Animals were kept separately in individual containers, the most satisfactory of which was a screw-capped gallon jar containing about an inch of water. A petri dish covered with paper toweling was placed in the bottom of the jar to provide a platform should the frog desire to exercise its amphibious urge to be out of water. Every two or three days the water was changed to eliminate accumulated wastes. On the day of sacrifice, pieces of ovary were fixed in Bouin's fluid containing 1% urea or in cold acetone. Routine staining was accomplished with Harris' hematoxylin and eosin, since this gave excellent contrast between the acidophilic platelets and the basophilic components of the cell. The triple stain of Slater and Dornfeld ('39) proved valuable for differentiating nucleoli, "yolk nuclei" and yolk platelets. Korson's ('51) technique was used to reveal localization of ribonucleoprotein, which stained blue with toluidin blue as contrasted with the yolk platelets, which stained yellow with Orange G. Taft's ('51) modification of Unna's combination of pyronin and methyl green gave results similar to those obtained with Korson's technique with respect to the distribution of ribonucleoprotein. Acetone-fixed material was used for

locating sites of localization of alkaline phosphatase by the Gomori technique. Slides were made from each ovary with about 20 adjacent sections mounted beneath long coverslips. These slides were scanned rapidly and the largest oocyte which appeared to be cut through the germinal vesicle was selected for measurement with a filar micrometer ocular. Because very few oocytes were exactly spherical, measurements were made of both the longest and shortest diameter. The results of the measurements made by this method of sampling are recorded in tables 1 and 2. Photographs were made with a Spencer photomicrographic camera utilizing 35 mm film.

I wish to acknowledge my appreciation for the painstaking assistance rendered by Miss Barbara L. Quinn in the technical aspects of this project.

RESULTS

A. Normal stages in deposition of yolk

Nieuwkoop ('49) adduces evidence which seems to support the view that the "primary" primordial germ cells in Amphibia are segregated early in embryonic development and that these cells through division give rise to all the reproductive cells of the adult. According to Gatenby ('16), however, all generations of oocytes in the amphibian ovary subsequent to the first arise by enlargement of cells derived directly from the germinal epithelium. The appearance of an ovary in an adult female sacrificed 23 days after ovulation is seen in figure 1. Three stages in enlargement of young oocytes are shown in figure 2. Chromatin in all of these cells is arranged in a network of fine strands which obviously elongate as the nucleus enlarges. In the nuclei of the three larger cells, several large nucleoli are visible beneath the nuclear membrane. Smaller nucleoli, probably still attached to chromosomes, are scattered throughout the nucleus. Cytoplasm in the smaller cells is only slightly basophilic with

hematoxylin, but there is increased basophilia in the larger cells. Irregular, basophilic bodies, the so-called "yolk nuclei," are also present in the cytoplasm of these larger cells. Preparations stained with Korson's or Unna's technique reveal that cells of similar size (40 μ) stain lightly with toludin blue or pyronin, indicating the accumulation of ribonucleic acid.

Duryee ('50) has published a valuable study on the normal growth of nuclei in oocytes of Rana pipiens. He distinguishes 6 stages as follows: (1) the smallest follicles containing germinal vesicles with visible chromosomes; (2) chromosomes paired in cells measuring less than 200 μ; (3) beginning of lateral loop production in cells 200 to 500 μ in diameter; (4) yellow-brown color indicating deposition of yolk and pigment in oocytes of 500 to 700 μ; (5) chromosome frame begins to contract, cells attaining 750 to 850 μ; (6) chromosome frame contracted to about one-thousandth of nuclear volume in mature oocytes measuring about 1.8 mm.

A pair of oocytes corresponding in age to Duryee's stage 1 are shown in figure 3. These cells, the larger of which measured 90 × 118 μ, protrude into the interior of the ovary and are surrounded by a follicular epithelium and theca interna. Both the number of nucleoli and the number and size of the "yolk nuclei" have noticeably increased from the values recorded for the cells in figure 2. The granular ground cytoplasm now stains deeply with hematoxylin or toluidin blue, indicating still further accumulation of ribonucleic acid. This intense basophilia continues through Duryee-stage 2 (fig. 4) when chromosomes have become paired. Nucleoli are still more abundant than in the previous stage, unquestionably because new ones have been synthesized and probably also because of fragmentation of older ones. "Yolk nuclei" are by now scattered throughout the cytoplasm.

By Duryee-stage 3, lateral loop production has begun on the chromosomes and the nucleus has begun to sacculate. Nucleoli are abundant, both peripherally and throughout the nucleus. An important observation for this stage is that the

"yolk nuclei" now seem concentrated toward the outside of the cytoplasm (fig. 5). The large cell in figure 5, measuring 175 × 192 μ falls slightly short of Duryee's estimate of 200 μ for the beginning of stage 3, but my measurements have been made on fixed material which has undoubtedly undergone some shrinkage.

Stage Y_0. A somewhat later stage (fig. 6), which would still be included in Duryee's stage 3 is classified as stage Y_0 in the present study; it is just prior to the deposition of yolk. One of the largest cells in this stage measured 286 × 352 μ. Cytoplasmic basophilia has now decreased and the "yolk bodies" appear to be concentrated in a ring around the nucleus. Of particular interest is the asymmetrical distribution of this ring, the basophilic bodies being obviously more abundant on one side of the nucleus. Peripheral to the perinuclear ring the ground cytoplasm is finely granular. Nucleoli by this stage appear to have decreased in number from the previous stage and are larger. Most of them are conspicuously located just beneath the nuclear membrane.

Stage Y_1. Yolk first appears at the periphery of an oocyte in the form of fine granules (figs. 7, 8). Coincidentally, a thin, striated layer, the "zona radiata," appears on the outer margin of the cell external to the ring of platelets; and shortly after deposition of yolk begins, the chorion can be detected, staining green with Korson's stain, as an exceedingly thin layer between the zona radiata and the follicular cells. Just inside the striated layer is a layer containing basophilic granules beautifully demonstrated with Korson's stain (fig. 9). Yolk platelets, staining yellow, evidently first appear within the blue basophilic layer and are pushed nuclead as new ones are formed. In later stages the thin basophilic layer persists external to the ring of yolk platelets. Brachet ('47a) first called attention to the basophilic cortex of amphibian oocytes and described a gradient of ribonucleic acid from animal to vegetative pole. It is clearly shown in figure 9 that this gradient becomes manifest from the time of earliest appearance of the basophilic cortex. Examination from sec-

tions of several animals failed to show any correlation between this cortical zone and the thickness of the ring of yolk platelets, for the latter was approximately uniform all around the cell. It is highly probable, however, that the gradient is closely related to deposition of pigment, which does not start until after stage Y_3 has been passed.

Stage Y_2. As mentioned above, new yolk platelets are formed at the periphery of the cell and those earlier formed are pushed inward toward the nucleus. Four specimens (table 1) were in stage Y_2 (fig. 10), i.e. with platelets extending inward approximately half the distance to the nuclear membrane. In figure 11 (also fig. 13) it can be seen that the older platelets increase in size as they move toward the nucleus. Once formed, platelets evidently serve as a center about which further material is added by accretion. Until approximately halfway in their inward march, the platelets remain closely packed in a ring. After this, however, individual platelets begin to move out ahead of the advancing ring and become widely dispersed throughout the perinuclear cytoplasm. Although most of these dispersed platelets are large and must have been pushed inward, a few small ones present suggest that new vitellogenesis may be starting in the interior cytoplasm. Throughout stage Y_2 there are many large nucleoli around the inside of the nuclear membrane, as well as many small ones scattered through the nucleoplasm.

Stage Y_3. Peripheral synthesis of yolk continues until platelets fill roughly three-fourths of the cytoplasm (figs. 12, 13). By this stage the largest platelets measure about 6 μ in diameter, the smallest about 0.8 μ. As in stage Y_2, scattered platelets may be seen in the perinuclear cytoplasm. The number of small, scattered platelets appears to have increased, lending further weight to the supposition that they were locally synthesized. The appearance of the nucleoli remains unchanged. During stage Y_3, fine pigment granules first appear at the periphery. From the start they are more abundant in the future animal hemisphere and are the first externally visible evidence of polarity. That they merely reflect

a preexisting polarity, however, seems evident from the observation mentioned above that granules containing ribonucleic acid are distributed apparently in animal-vegetative polarity even earlier.

Stages Y_4 and Y_5. Stage Y_4 is reached when yolk platelets fill virtually all of the cytoplasm, with the exception of the cortical zone previously mentioned. Data available indicate that oocytes reach a diameter of approximately 850 μ before attaining stage 4. Considerable growth ensues after this size is reached until the oocyte becomes full-grown, after which it may be classified in stage Y_5 (fig. 14). It will be noted that stage Y_4, as described here, corresponds to a stage intermediate between Duryee's stages 5 and 6, while stage Y_5 in the present study is the same as Duryee's stage 6. Five experimental specimens (table 1) reached stage Y_4 and most of the animals developing in nature until the end of July, August or September had reached stage 4; one in August and several in September had even reached stage Y_5. Wittek ('52) distinguishes between primary and late vitellogenesis, the former referring to the early centripetal deposition of platelets as a result of peripheral synthesis, the latter to a centrifugal accumulation of platelets around the nucleus. The period from our stage Y_3 to stage Y_5 would roughly embrace Wittek's "vitellogénèse tardive."

B. Comparative rate of deposition of yolk in laboratory-fed animals

Before the investigation actually got under way, it was anticipated that there would be a fair degree of uniformity of rate of synthesis of yolk subsequent to ovulation in animals kept under nearly-identical laboratory conditions. As it turned out, there was indeed a general correlation between amount of yolk deposited and post-ovulatory age, but time to reach particular stages varied considerably. Data on size of oocytes, post-ovulatory age, and deposition of yolk and pigment are assembled in table 1. Because synthesis of yolk was much

faster in the animals of the 1952 series, they are distinguished from those of the 1951 series by asterisks.

From table 1 it is readily seen that three specimens of the 1951 series, respectively 9, 23 and 40 days in post-ovulatory

TABLE 1

Deposition of yolk and pigment in growing oocytes of Rana pipiens at various ages after ovulation

STAGE	PROTOCOL NUMBER	DAYS AFTER OVULATION	DIAMETER IN MICRONS
Y_0	17	9	279 × 290
	24	23	290 × 334
	80	40	366 × 437
Y_1	*108	1	370 × 486
	67	30	356 × 515
	87	30	393 × 462
	65	58	409 × 467
Y_2	*98	28	485 × 567
	*101	39	518 × 566
	76	40	438 × 570
	77	52	601 × 706
	64	59	510 × 648
	23	90	485 × 590
Y_3	*104	19	606 × 700
	74	29	500 × 621
	*96	32	486 × 678
	*103	44	550 × 552
	72	55	735 × 820
	85	60	658 × 719
Y_3, plus pigment	*106	25	740 × 851
	*107	25	1120 × 1235
	88	53	661 × 810
	90	60	848 × 952
	78	60	751 × 926
	33	66	800 × 849
	70	75	850 × 865
Y_4	34	90	907 × 1019
	38	90	995 × 962
	*94	118	1150 × 1260
	*100	118	1290 × 1322
	*102	118	1233 × 1280
Y_5			1390 × 1675

Y_0 — No yolk.
Y_1 — Thin ring of yolk at periphery.
Y_2 — Yolk half way from periphery of cell to nucleus.
Y_3 — Yolk three-quarter way from periphery of cell to nucleus.
Y_4 — Yolk completely filling cytoplasm.
Y_5 — Mature primary oocyte (from a non-ovulated control in May, 1952).
* — Specimens marked with asterisk in 1952 series; rest in 1951 series.

age, possessed no yolk whatsoever. Three specimens of the 1951 series, two aged 30 days and one of 58 days, had reached stage Y_1; but one animal of the 1952 series examined only one day after stripping had also reached stage Y_1. Presumably it had already started to deposit yolk in young oocytes even before ovulation. The most plausible explanation for such variability is the state of nutrition of individual females. It was noted that females of the 1952 series looked particularly vigorous and healthy when received from the supplier. It is possible too that as an animal gets closer to the breeding season it might take less time for yolk to appear than in an animal earlier in the year. Specimen 108, which showed precocious deposition of yolk, however, ovulated in February, whereas some other animals ('51 series), which took longer to deposit yolk, ovulated in April or May.

Six specimens were classified in stage Y_2. These included 4 from the 1951 and 2 from the 1952 group, ranging from 28 to 90 days in post-ovulatory age. Six more, 3 from the 1951 series and 3 from 1952, had reached stage Y_3. Ages varied from 19 to 60 days. Although the yolk was in stage Y_3 in 7 additional specimens, they are classified separately because pigment was also present. Five specimens, varying from 53 to 75 days of post-ovulatory age, reached this condition in the 1951 group; 2 of the 1952 group, however, had reached it only 25 days after ovulation. Five specimens, 2 fed for 90 days and 3 for 118 days after ovulation, are classified in stage Y_4. By this time pigment had increased and the oocytes grown so that animal and vegetative hemisphere were clearly distinguishable. None of these animals reached the size of a mature oocyte (stage Y_5, table 1), but it is probable that they would have with longer feeding.

C. Comparative rate of deposition of yolk in animals collected from nature

In order to compare rate of deposition of yolk in laboratory-fed animals with that in animals feeding in the wild, a dozen

large, mature female frogs were ordered from the supplier in each of the months of July, August and September, 1952, with the stipulation that they be collected from nature after receipt of the order or have had an opportunity to feed naturally since the spring breeding season. These animals were sacrificed on July 28, August 28, and September 29, 1952, shortly after receipt in the laboratory. Data on stages and sizes of the largest oocytes observed in these animals are shown in table 2.

TABLE 2

Deposition of yolk in frogs collected from nature in July, August and September

JULY		AUGUST		SEPTEMBER	
Stage	Diameter in microns	Stage	Diameter in microns	Stage	Diameter in microns
Y_2	486 × 486	Y_4	1132 × 1132	Y_0	263 × 344
Y_3	615 × 714	Y_4	1213 × 1260	Y_0	324 × 324
Y_4	850 × 870	Y_4	1100 × 1295	Y_4	1038 × 1100
Y_4	880 × 900	Y_4	1255 × 1325	Y_4	1130 × 1168
Y_4	933 × 980	Y_4	1135 × 1330	Y_4	1176 × 1392
Y_4	648 × 1000	Y_4	1332 × 1332	Y_5	1440 × 1440
Y_4	835 × 1000	Y_4	1330 × 1352	Y_5	1457 × 1457
Y_4	1050 × 1050	Y_4	1150 × 1380	Y_5	1443 × 1475
Y_4	1019 × 1130	Y_4	1293 × 1390	Y_5	1488 × 1536
Y_4	1080 × 1175	Y_4	1370 × 1410	Y_5	1438 × 1538
Y_4	1090 × 1185	Y_5	1241 × 1422	Y_5	1535 × 1620
Y_4	1165 × 1260	Y_4	1245 × 1440	Y_5	1540 × 1620

The table shows that 10 of the 12 frogs received in July had reached stage Y_4 and that representative oocytes in these specimens varied in size from 850 × 870 μ to 1165 × 1260 μ (average, 955 × 1055 μ). By the end of August there was noticeable additional growth with oocytes in individual specimens ranging from 1132 × 1132 μ to 1245 × 1440 μ (average, 1233 × 1339 μ). One specimen, measuring 1241 × 1422 μ, was classified in stage Y_5 because of the advanced state of contraction of the chromosome frame. The rest, rated in stage Y_4, had only partially contracted chromosome frames. By the end of September, 7 specimens had well-contracted chro-

mosome frames and were therefore classified in stage Y_5. Disregarding the two specimens in stage Y_0, we now find oocytes from 1038 × 1100 μ to 1540 × 1620 μ (average, 1370 × 1435 μ). From these measurements it seems safe to conclude that oocytes which have reached a size in the vicinity of 1400 μ could be classified in stage Y_5. It should be noted that these figures apply only to fixed and sectioned material. On this basis, we may consider specimen 100 (table 1), maintained for 120 days in the laboratory, to have attained thirteen-fourteenths (approximately 93%) of full growth.

DISCUSSION

In the present study it has been shown that deposition of yolk in the oocytes of adult females of Rana pipiens is initiated when tiny platelets appear just beneath the cortical cytoplasm. The time of appearance of yolk varies (cf. specimens in stages Y_0 and Y_1, table 1), probably depending on the nutritive state of the particular animal at the time of ovulation. If the female is well-fed and healthy, synthesis of yolk apparently may begin even before ovulation (specimen 108, table 1). In less robust animals, however, synthesis of yolk may be delayed. Subsequent to their initial deposition, platelets continue to form at the periphery, while those earlier formed increase in size and move inward toward the nucleus. By this means an increasingly broad peripheral ring of yolk is deposited. After this ring extends approximately half of the distance toward the nucleus, scattered platelets appear in the perinuclear cytoplasm, including some tiny ones evidently newly synthesized in this region. Pigment now appears in the form of tiny granules deposited just beneath the cortical cytoplasm. This stage was not attained in animals of the 1951 series until 53 days after ovulation (specimen 88, table 1). In the 1952 series, however, pigment was present by 25 days (specimens 106 and 107, table 1). It is important to realize that from the time of its first appearance pigment is more abundant at one pole, henceforth distinguished as the animal pole, than at the other. As the oocytes enlarge further, yolk

comes to fill the previously yolk-free perinuclear cytoplasm so that eventually the entire cytoplast, except for the cortex, is filled. Daniel and Yarwood ('39) have reported a gradient in average size of platelets from animal toward the vegetative pole in fully grown oocytes of Triturus torosus, and Bragg ('39) has described a similar gradient in the cells of cleaving embryos of Rana pipiens. Wittek ('52) reports that in Rana temporaria, Triturus helveticus, Triturus alpestris, and Xenopus laevis at the end of vitellogenesis one may distinguish three main regions with respect to size of platelets — a zone of small platelets capping the animal hemisphere, a cup-shaped mass in the vegetative hemisphere containing large platelets, and a central region containing the nucleus and filled with medium-sized platelets. Pasteels ('46) describes these three regions also in the axolotl but adds the region of the maturation spindle as a 4th zone of the egg.

One of the great unsolved mysteries of oogenesis is the origin of polarity. Even if we accept Child's well-known explanation of metabolic gradients to account for the first visible polarity, we are still faced with ignorance of the nature of the metabolites involved. In the frog's egg differential deposition of pigment is the first external indication of polarity, although the displacement of the germinal vesicle toward one pole, seen in sectioned material, constitutes a polarity established earlier. Wittek ('52) finds that the axis determined by nuclear displacement does not necessarily coincide with that established by pigment-deposition. He holds, therefore, that bilateral symmetry may already be apparent when pigment is first deposited. Brachet ('47a) has demonstrated a cortical gradient of ribonucleoprotein in amphibian oocytes. This gradient, easily visualized with toluidin blue or pyronin, has been shown in the present study to appear even prior to the deposition of pigment. Wittek ('52, p. 146) describes a similar gradient of distribution of pyronin-positive cytoplasm in a young oocyte of Triturus alpestris. There is considerable evidence that cytoplasmic ribonucleoproteins are the products of nucleolar substance which is in some manner transferred

through the nuclear membrane. A number of authors (discussion by Wittek, '52) claim to have histological preparations showing the passage of intact nucleoli through the membrane, but the majority of workers have seen nothing which convincingly proves the direct transfer of nucleoli through the nuclear membrane. In the present study it has been observed that early stages in growth of the oocytes show first an increase in number of nucleoli and their concentration just beneath the nuclear membrane (figs. 2–5). While this increase continues, basophilic cytoplasmic masses appear and become concentrated around the periphery of the cell. These bodies in no way resemble the nucleoli except in staining properties. Subsequently, the basophilic bodies take up a position in a perinuclear ring deep within the cytoplasm (fig. 6). Shortly after this stage is reached, yolk is first deposited and the cortical zone, already polarized in its staining reaction with toluidin blue or pyronin, appears. Assuming that the cortical ribonucleoproteins are derived from migrating nucleolar substance, we must explain their polarized distribution, (1) as the result of an intrinsic polarity already present in the cortical cytoplasm before the cortical basophilic layer appears, or (2) the result of a polarizing influence in the follicle. In favor of the first explanation is the observation recorded by Wittek ('52) that the nucleus early becomes eccentric in position, since this indicates that the cytoplasm has developed polarity.

It is well established from the work of Caspersson ('41) and Brachet ('41, '47a) that ribonucleoprotein accumulates in cells undergoing active synthesis of protein. Just how ribonucleoproteins function in the synthesis of yolk platelets remains to be determined. According to Brachet ('50, p. 333), amphibian yolk is chiefly protein, probably associated with lipids. It contains both phosphoprotein and nucleoprotein. Harris ('46) discovered an enzyme, phosphoprotein phosphatase (PPPase), associated with the yolk platelets and Panijel ('50) has demonstrated that small platelets have more PPPase activity than large ones. It might be expected from

the observation that the larger yolk platelets are the oldest and have reached their size by gradual growth, that they would have a different chemical composition from the smaller and younger platelets. An important agent in synthesis of yolk, apparently, is the enzyme dipeptidase described by Duspiva ('42). This enzyme was shown to increase rapidly at about the time of beginning deposition of yolk and to decrease as yolk accumulated. Pickford ('43) believes that this same enzyme is also involved in the finer phases of intracellular digestion of yolk during embryogenesis. Brachet ('47b) has suggested that alkaline phosphatase in the nucleus might be involved in the synthesis of yolk, but Osawa ('51) on the basis of histochemical studies has obtained evidence which, he believes, casts some doubt on this hypothesis. If one tests sections of developing oocytes by the usual Gomori technique for alkaline phosphatase, it is found that nuclei of follicle cells, nucleoli of oocytes, and yolk platelets from the time of their earliest appearance (personal observation) are blackened. According to Osawa ('51), however, the staining observed in nucleoli, nuclear sap and yolk granules is merely an artifact due to diffusion. He also points out that the use of cobalt in the Gomori technique is inappropriate for amphibian embryos because of the specific affinity of nucleoli and yolk platelets for this element. He used Van Kossa's procedure for visualization of calcium phosphate, which involves treatment with silver nitrate and exposure to light after incubation, followed by treatment with sodium thiosulfate (hypo). Further work needs to be done to clarify the role (if any) of alkaline phosphatase in synthesis of yolk.

Ultimately we should like to understand all of the chemical reactions involved in synthesis of yolk, as well as its utilization during embryonic development. Brachet ('50, p. 57) emphasizes the need for the development of new methods in stating that "cytochemical studies can teach us nothing of the chemical mechanism of these syntheses, they show us only the topographical localization of the substances determined." We agree heartily that new methods are needed but would

recommend to investigators in this field that they be forearmed with all known or determinable cytological details concerning the sequence and rate of deposition of yolk.

SUMMARY

1. Stages in the growth of oocytes of Rana pipiens both prior to and during deposition of yolk are described. Before vitellogenesis begins, nucleoli increase in number and concentrate beneath the nuclear membrane. While this is going on, basophilic bodies called "yolk nuclei" appear in the cytoplasm, move to the periphery, then become concentrated in a perinuclear ring at stage Y_0.

2. Shortly after induced ovulation, yolk is first deposited in a thin peripheral ring (stage Y_1) in some of the small oocytes remaining in the ovary. Deposition of yolk may already have started by the time of ovulation (one case) or it may be delayed for over a month, probably depending on the nutritive state of the particular animal. Coincident with the deposition of yolk, a cortical zone exhibiting polarity in distribution of ribonucleic acid appears.

3. Yolk continues to form peripherally while platelets earlier formed enlarge as they are pushed inward toward the nucleus. Stage Y_2 is reached when the peripheral ring of yolk occupies about one-half of the cytosome. Scattered platelets may be seen between the peripheral ring and the nucleus.

4. Stage Y_3 is reached when the peripheral ring of platelets fills three-fourths of the cytosome. Shortly thereafter pigment granules first appear at the periphery of the animal pole.

5. Stage Y_4 is characterized by filling of the entire cytosome with yolk. This stage was attained in laboratory-fed frogs after 90 days and in 10 out of 12 animals collected from nature late in July.

6. Oocytes, though still in stage Y_4, showed increased growth in two animals maintained in the laboratory for 118 days after ovulation. Stage Y_5 (the mature oocyte) was

reached in one animal collected in nature late in August and in 7 of 12 animals toward the end of September.

7. The origin of polarity in the oocyte and the mechanism of synthesis of yolk are discussed.

LITERATURE CITED

BRACHET, J. 1941 La détection histochimique et le microdosage des acides pentosenucléiques (tissus animaux — développement embryonnaire des Amphibiens). Enzymologia, *10:* 87–96.

———— 1947a The metabolism of nucleic acids during embryonic development. Cold Spring Harbor Symp. on Quant. Biol., *12:* 18–27.

———— 1947b Biochemical and physiological interrelations between nucleus and cytoplasm during embryonic development. Growth Symp., *11:* 309–324.

———— 1950 Chemical embryology. Interscience Publ., New York.

BRAGG, A. N. 1939 Observations upon amphibian deutoplasm and its relation to embryonic and early larval development. Biol. Bull., *77:* 268–284.

CASPERSSON, T. 1941 Studien über den Eiweiszumsatz der Zelle. Die Naturwissenschaften, *29:* 33–43.

DANIEL, J. F., AND E. A. YARWOOD 1939 The early embryology of Triturus torosus. Univ. Calif. Pub. Zool., *43:* 321–356.

DURYEE, W. R. 1950 Chromosomal physiology in relation to nuclear structure. Ann. N. Y. Acad. Sci., *50:* 920–953.

DUSPIVA, F. 1942 Die Verteilung der Peptidase auf Kern und Plasma bei Froschoozyten im Verlauf der zweiten Wachstumsperiode. Biol. Zentralbl., *62:* 403–431.

EAKIN, R. M., P. B. KUTSKY AND W. E. BERG 1951 Protein metabolism of amphibian embryo. III. Incorporation of methionine into protein of gastrulae. Proc. Soc. Exp. Biol. and Med., *78:* 502–504.

FISCHER, I. 1932 Beiträge zur Kenntnis des Jahreszyklus des Urodeleneierstockes. Teil I. Die Wachstumsperiode des Eifollikels, der sprungreife Follikel und der Narbenfollikel von Triton alpestris. Zeit. f. mikr. Anat. Forsch., *31:* 425–520.

FRIEDBERG, F., AND R. M. EAKIN 1949 Studies in protein metabolism of the amphibian embryo. I. Uptake of radioactive glycine. J. Exp. Zool., *110:* 33–46.

GATENBY, J. B. 1916 The transition of peritoneal epithelial cells into germ cells in some Amphibia Anura, especially in Rana temporaria. Quart. J. Micr. Sci., *61:* 275–300.

GATENBY, J. B., AND J. H. WOODGER 1920 On the relationship between the formation of yolk and the mitochondria and Golgi apparatus during oögenesis. J. Roy. Micr. Soc., *151:* 129–156.

HARRIS, D. L. 1946 Phosphoprotein phosphatase, a new enzyme from the frog egg. J. Biol. Chem., *165:* 541–550.

HIBBARD, H. 1928 Contribution à l'étude de l'ovogenèse, de la fécondation, et de l'histogenèse chez Discoglossus pictus otth. Arch. de Biol., *38:* 251–326.

HOLTFRETER, J. 1946 Experiments on the formed inclusions of the amphibian egg. I. The effect of pH and electrolytes on yolk and lipochondria. J. Exp. Zool., *101:* 355–405.

JÖRGENSEN, M. 1913 Zellenstudien. I. Morphologische Beiträge zum Problem des Eiwachstums. Arch. f. Zellforsch., *10:* 1–126.

KING, HELEN D. 1908 The oögenesis of Bufo lentiginosus. J. Morph., *19:* 369–438.

KORSON, R. 1951 A differential stain for nucleic acids. Stain Tech., *26:* 265–270.

LAMS, H. 1907 Contribution à l'étude de la genèse du vitellus dans l'ovule des Amphibiens. Arch. d'Anat. Micr., *9:* 607–663.

NIEUWKOOP, P. D. 1949 The present state of the problem of the "Keimbahn" in the vertebrates. Experientia, *5:* 308–312.

OSAWA, S. 1951 Histochemical studies of alkaline phosphatase in the oogenesis and the early embryogenesis of the Amphibia. Embryologia, *2:* 1–20.

PANIJEL, J. 1950 L'organisation du vitellus dans les oeufs d'Amphibiens. Biochem. et Biophys. Acta, *5:* 343–357.

PASTEELS, J. 1946 Sur la structure de l'oeuf insegmenté d'axolotl et l'origine des prodromes morphogénétiques. Acta Anat., *2:* 1–16.

PICKFORD, G. E. 1943 The distribution of dipeptidase in the salamander gastrula. J. Exp. Zool., *92:* 143–170.

RUGH, R. 1937 A quantitative analysis of the pituitary-ovulation relation in the frog (Rana pipiens). Physiol. Zool., *10:* 84–100.

———— 1948 Experimental embryology. Burgess Publ. Co., Minneapolis.

RYAN, F. J., AND R. GRANT 1940 The stimulus for maturation and for ovulation of the frog's egg. Physiol. Zool., *13:* 383–389.

SLATER, D. W., AND E. S. DORNFELD 1939 A triple stain for amphibian embryos. Stain Tech., *14:* 103–104.

TAFT, E. B. 1951 The problem of a standardized technic for the methyl-green-pyronin stain. Stain Tech., *26:* 205–212.

WITTEK, M. 1952 La vitellogénèse chez les Amphibiens. Arch. de Biol., *63:* 133–198.

WORLEY, L. G. 1943 The structure and function of the Golgi system in the living cells of developing molluscs. Proc. Nat. Acad. Sci., *29:* 225–228.

———— 1944 Studies of the vitally stained Golgi apparatus. II. Yolk formation and pigment concentration in the mussel Mytilus californianus Conrad. J. Morph., *75:* 77–101.

PLATE 1

EXPLANATION OF FIGURES

1 Low-power view of section of ovary of specimen Ov-24, fixed 23 days after ovulation. Oocytes are in pre-yolk stages. Black masses are sites of retrogression of follicles which matured in previous cycle. Slater and Dornfeld's triple stain. × 50.

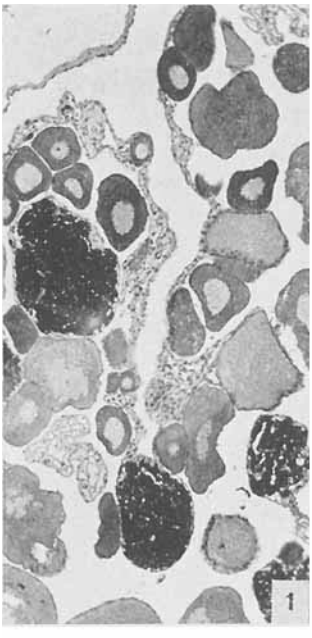
2 Enlarging oocytes in specimen Ov-24. Nucleoli are conspicuous beneath nuclear membrane in three larger cells, the largest of which measured 40.4 μ in its longest diameter. Nuclei of two smaller stages are seen in cells to the right of the upper two in the row of larger cells. Slater and Dornfeld's triple stain. × 500.

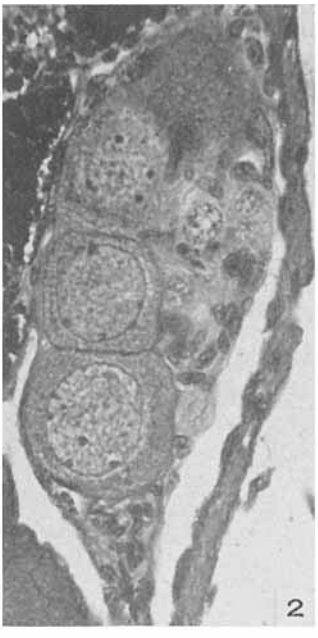
3 Two oocytes from specimen Ov-24 in Duryee-stage 1, now surrounded by follicle cells and protruding into lumen of ovary. Larger cell measured 90 × 118 μ. Slater and Dornfeld's triple stain. × 230.

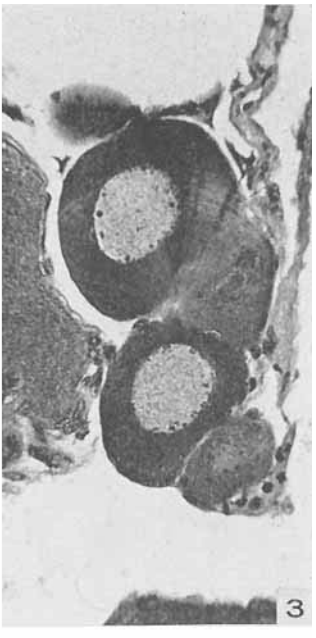
4 Two oocytes from specimen Ov-24 in Duryee-stage 2. Note increase in number of nucleoli beneath nuclear membrane. Larger cell measured 126 × 176 μ. Slater and Dornfeld's triple stain. × 230.

5 Two oocytes from specimen Ov-24 in Duryee-stage 3. Chromosomes have started to form lateral loops and nuclear membrane has begun to sacculate. "Yolk nuclei" are concentrated peripherally in the cell. Larger cell measured 175 × 192 μ. Slater and Dornfeld's triple stain. × 170.

PLATE 1







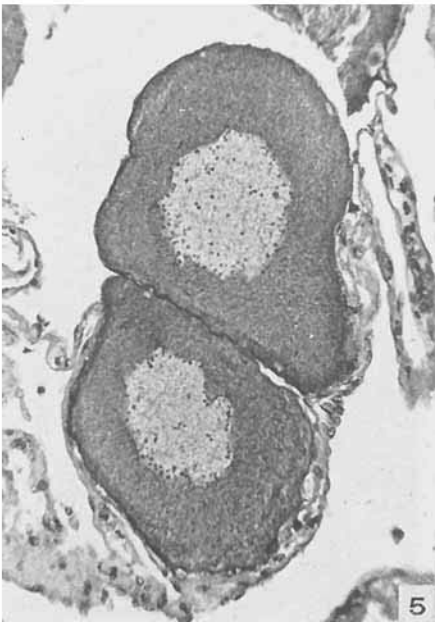


PLATE 2

EXPLANATION OF FIGURES

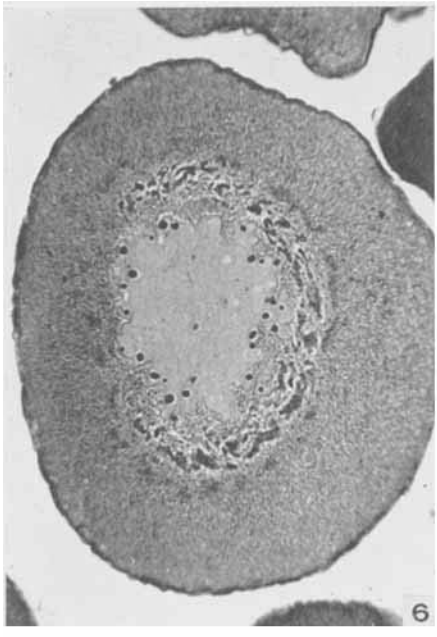
6 Stage Y_0, just prior to deposition of yolk in specimen Ov-24. Note large nucleoli next to nuclear membrane and asymmetrical accumulation of "yolk nuclei" around the nucleus. Cell measured 286 × 352 μ. Slater and Dornfeld's triple stain. × 195.

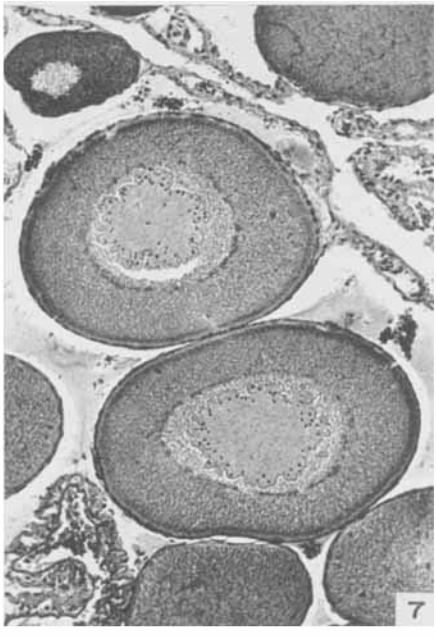
7 Stage Y_1 in specimen Ov-108 (fixed one day after ovulation) just subsequent to initial deposition of yolk at the periphery. Note ring of "yolk nuclei" just outside the light perinuclear cytoplasm. Size, 322 × 436 μ. Hematoxylin and eosin. × 85.

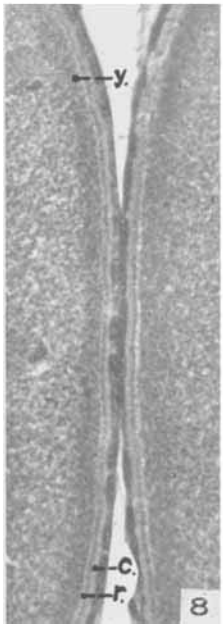
8 Enlargement of adjacent surfaces of oocytes shown in figure 7. Follicle cells are in direct contact. Just beneath these is the light chorion (c.), which is closely applied against the "zona radiata" (r.), the striated layer on the surface of the oocyte. The dark layer internal to this (y.) is composed of tiny yolk platelets. Hematoxylin and eosin. × 850.

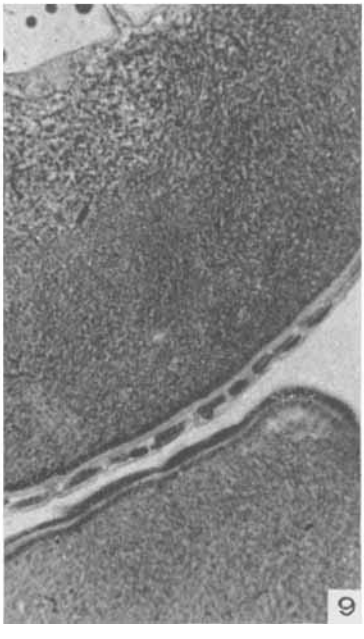
9 Specimen Ov-67 (fixed 30 days after ovulation) at stage Y_1 shows early polarity in distribution of ribonucleic acid in cortical layer which is toluidin-blue positive. Region where concentration of ribonucleic acid diminishes (following from left to right in upper cell) probably indicates boundary between animal and vegetative poles. Korson's stain. × 850.

10 Stage Y_2 from specimen Ov-74 fixed 29 days after ovulation. Yolk platelets extend approximately one-half of the distance from cortex to nucleus. Cell measured 454 × 567 μ. Hematoxylin and eosin. × 85.


6


7


y.
c.
r.
8


9

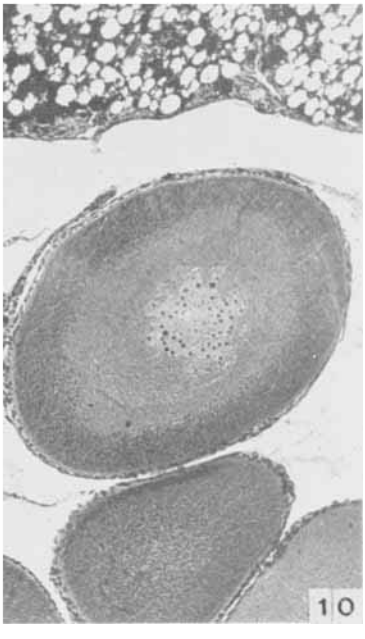

10

PLATE 3

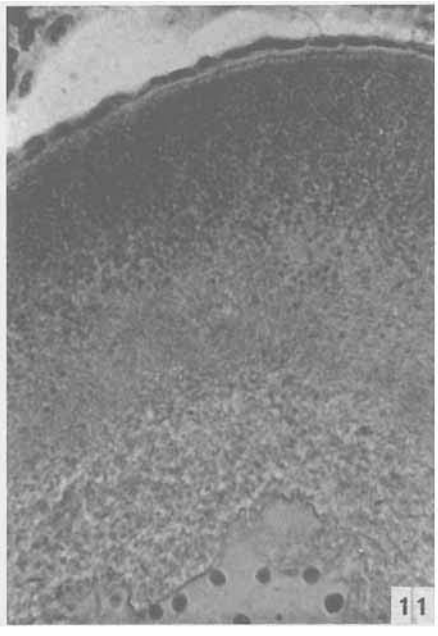
EXPLANATION OF FIGURES

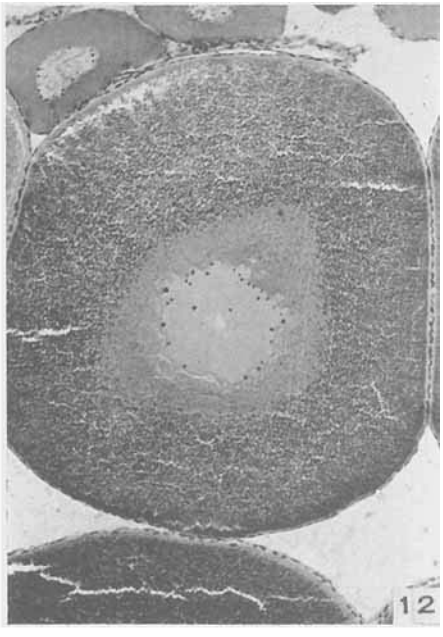
11 Enlargement of section of oocyte from specimen Ov-74 in stage Y_2. Growth of platelets as they move toward the nucleus is evident. A few scattered platelets may be seen in the vanguard of the advancing peripheral ring. Hematoxylin and eosin. × 850.

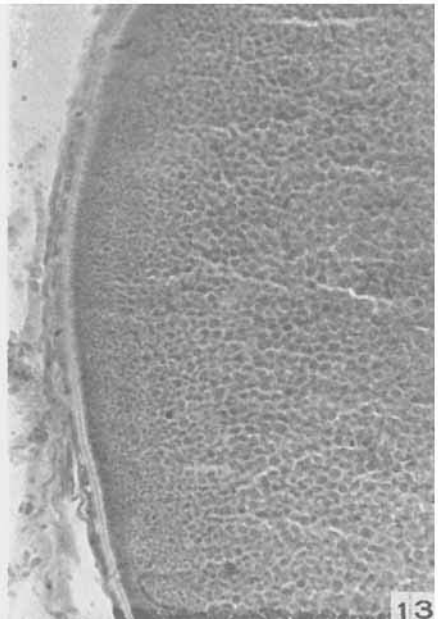
12 Stage Y_3 from specimen Ov-72, 55 days after ovulation. Yolk platelets extend approximately three-fourths of the distance from cortex to nucleus. Size, 648 × 710 μ. Hematoxylin and eosin. × 85.

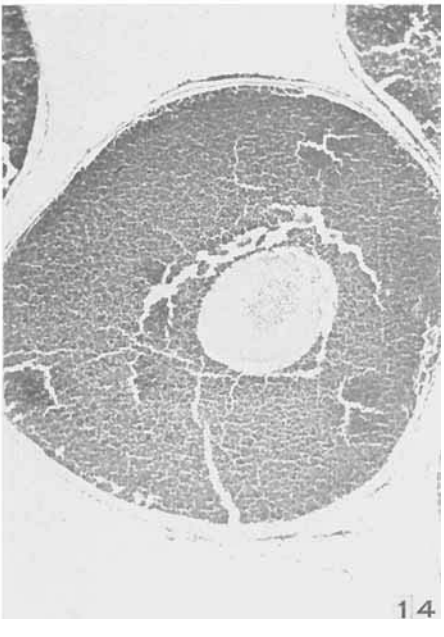
13 Enlargement of periphery of oocyte from specimen Ov-72 in stage Y_3. Growth of yolk platelets from the periphery toward the interior is obvious. Hematoxylin and eosin. × 850.

14 Stage Y_5, measuring 1275 × 1400 μ, from an unovulated ovary fixed in May, 1952. Yolk fills entire cytoplasm and nucleoli are clustered about the contracted chromosome frame. Space between nucleus and yolk is an artifact due to shrinkage. Hematoxylin and eosin. × 42.5.


11


12


13


14