NUCLEIC ACID AND PROTEIN SYNTHESIS IN THE DEVELOPING OOCYTES OF THE BUDDING FORM OF THE SYLLID, AUTOLYTUS EDWARSI (CLASS POLYCHAETA) [1]

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In recent years the importance of the role of deoxyribonucleic acid (DNA) and the various ribonucleic acids in protein synthesis and the significance of the origin of new proteins in cellular differentiation have become increasingly clear. It now seems an accepted fact that the nucleus is the major site of ribonucleic acid (RNA) synthesis in a wide variety of cell types, including developing oocytes (Ficq, 1961; Ficq, *et al.*, 1963; Favard-Séréno and Durand, 1963a). However, as far as intranuclear metabolism is concerned, the situation is far from clear. Favard-Séréno and Durand (1963a) observed that the chromosomes are the primary site of RNA synthesis in follicle cells of the cricket ovary; from its source RNA then migrates to the nucleolus where it accumulates before moving to the cytoplasm. Presumably the nucleolus in these cells is serving simply as a temporary storage depot for RNA. However, for a wide variety of cells, and this includes a number of types of developing oocytes (for example, Ficq, 1953, 1955a, 1955b; Edström, 1960; Edström *et al.*, 1961), the conclusion has been that the nucleolus is an important site of RNA synthesis providing a major portion of cytoplasmic RNA.

One approach to the problems of whether the nucleolus is simply accumulating RNA and whether or not RNA synthesis is correlated with DNA and protein synthesis in developing eggs has been the use of radioautography. Various investigators (for example, Brachet and Ficq, 1956; Ficq, 1959) have employed specific radioactive precursors to determine the site of synthesis of RNA, DNA, and proteins within developing oocytes, and to follow the subsequent migration of these macromolecules. The oocytes thus far investigated have been mainly those of amphibians (Ficq, 1955a; Ficq *et al.*, 1958; Pantelouris, 1958; Gall, 1963; Ozban *et al.*, 1964), echinoderms (Ficq, 1955b, 1962; Geuskens, 1963, 1965), and insects (King and Burnett, 1959; Nigon and Nonnenmacher, 1961; Favard-Séréno and Durand, 1963a, 1963b; Vanderberg, 1963), and, more recently, of an echiuroid worm (Das *et al.*, 1965), and two species of polychaetous annelids (Tweedell, 1964, 1966; Allen, 1966).

The developing oocytes of the polychaete, *Autolytus edwarsi*, which were used in the present investigation, incorporate uridine-H^3 with unusual rapidity (Allen, 1966), and also incorporate phenylalanine-H^3. It was thus felt that the details of the results on *Autolytus* eggs should be reported, and particularly in view of

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the fact that varying results have been reported as to the site of RNA and protein synthesis in unfertilized eggs.

The following description will serve as background for the radioautographic study. The writer recently has pointed out that "The Syllidae are unusual among the polychaetes in that some genera of this family reproduce both asexually by the formation of stolons from a parent stock, and sexually by the union of gametes from male and female individuals which, in turn, originate from stolons produced asexually" (Allen, 1964, page 187). The genus *Autolytus* is a good example of this type of life cycle. The eggs of the budding form of *A. edwarsi* develop in a rather unusual manner (Gidholm, 1963). Gidholm confirmed Meyer's discovery (1914) that ovaries occur only in the stock of *Autolytus* yet the whole chain of stolons, even the undifferentiated ones, may contain well developed eggs. Gidholm points out that Meyer "correctly interpreted these facts to mean that the eggs are not formed in the stolons but rather in the posterior part of the stock, from where they are transported to the stolons" (Gidholm, 1963, page 529). According to Gidholm, the oocytes are released from the ovary, not into the coelom as Meyer had thought, but into an epithelial-lined oocyte cavity which is continuous from the ovaries of the stock through the region of proliferation to the budding stolons. By vital staining of the oocytes, Gidholm was able to follow migrating eggs from the oocyte cavity of the stock through the region of proliferation where the cavity narrows to a canal, and then into the differentiating stolons in which the continuous oocyte cavity enlarges again (Gidholm, 1963, page 533, Figs. 1 and 2, and page 538).

The budding form of *A. edwarsi* with its linear chain of successively differentiating stolons provides an ideal place for the study of cellular differentiation; it is small enough so that in the present study the whole organism (stock with attached stolons) was treated with labeled precursors for nucleic acids and proteins. Since the oocytes are the largest cells and thus lend themselves to more accurate cytological analysis than do the other cells, the results presented first are those demonstrating incorporation of labeled precursors into developing eggs.

The results indicate that RNA is synthesized (or accumulated) in the nucleolus of the developing oocytes from which it migrates to the rest of the nucleus and then to the cytoplasm (also see Allen, 1966). Protein synthesis, on the other hand, appears to occur throughout the developing oocyte (*i.e.*, in the nucleolus, the nonnucleolar regions of the nucleus, and the cytoplasm). There was no evidence of incorporation of thymidine into the developing oocytes, indicating the absence of any appreciable DNA synthesis.

Material and Methods

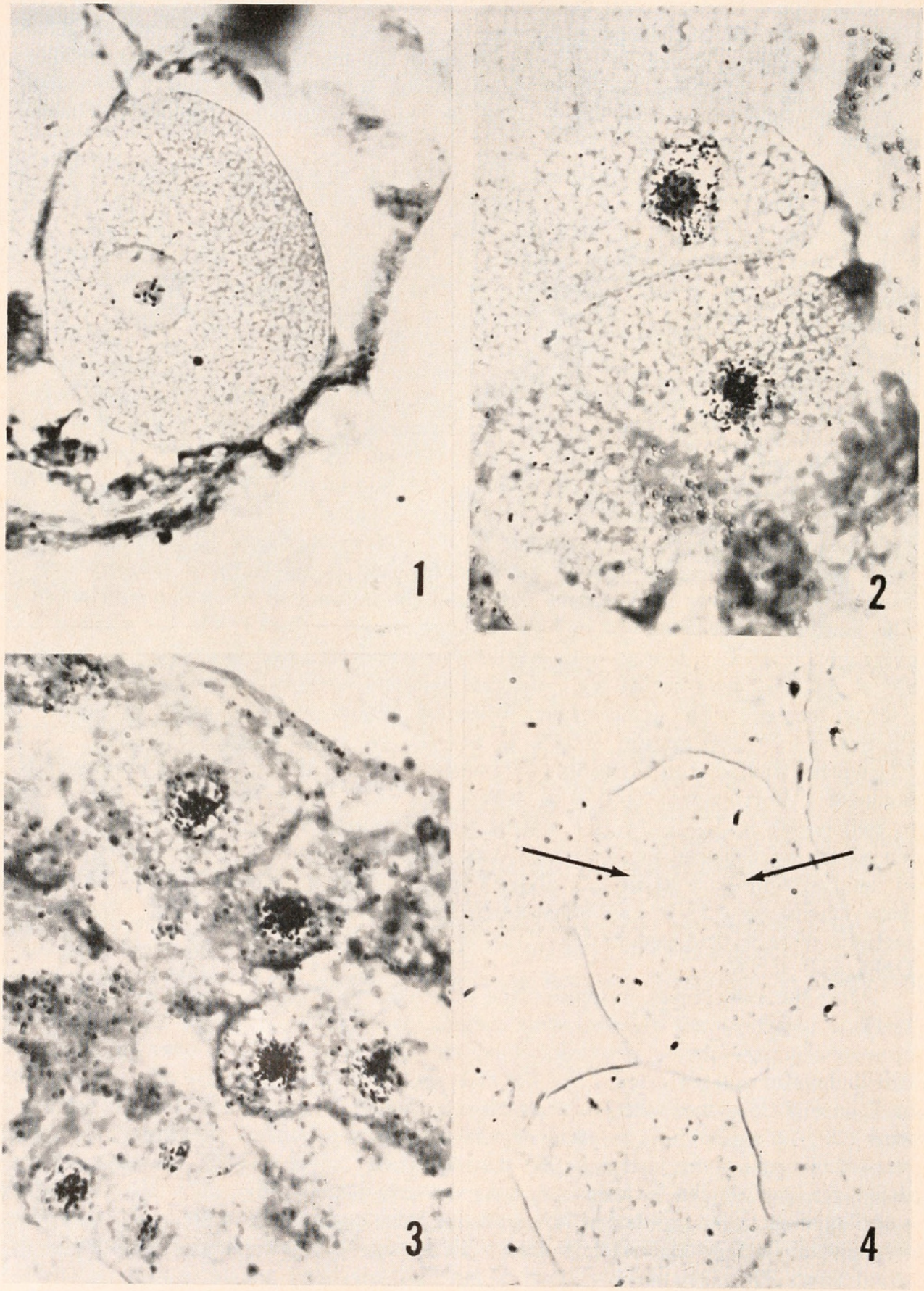
The budding form of *Autolytus edwarsi* lives in association with the hydroid, *Laomedea* (*Obelia*) *geniculata*, which in turn, is found abundantly on the brown alga, *Laminaria*. Stocks of *A. edwarsi* with linear chains of stolons, and stocks which were not obviously budding, were kept until needed in small covered glass dishes supported on a perforated tray suspended in the sea water aquarium. Preliminary experiments in which stocks with their attached stolons were immersed in the isotope solutions indicated that these multicellular organisms were small enough so that very short periods of treatment were sufficient to permit diffusion

of radioisotopes through their body wall. Tritiated uridine, thymidine, and phenylalanine were used to determine the site of synthesis (or accumulation) of RNA, DNA, and protein, respectively, in the tissues of the stock and differentiating stolons of *A. edwarsi*. The treatment with radioisotopes, subsequent fixation of the worms, and embedding in paraffin were done at the Kristineberg Zoological Station, Sweden, in the fall of 1963.

In general, 10 stocks with their differentiating stolons were treated at a time in one milliliter of a solution of radioisotope in filtered sea water. During exposure to the isotope, the cover of the glass dish in which they were treated was lined with a layer of filter paper moistened with distilled water to prevent evaporation and maintain the proper humidity. Worms were immersed thus for periods varying from 2 minutes to 20 hours in one of the following radioisotopes: 2.5 μc./ml. of uridine-H^3 (0.90 c./m*M*, Schwarz Bioresearch, Inc.), 2.5 μc./ml. of thymidine-H^3 (6.05 c./m*M*, Schwarz Bioresearch, Inc.) or approximately 2.5 μc./ml. of DL-phenylalanine-H^3 (33.2 mc./m*M*, New England Nuclear Corporation). In early experiments, following exposure to the isotope, the worms were washed in several changes of sea water (200 ml. for each change), but in later experiments they were placed in an excess of non-labeled precursor, *i.e.*, uridine, thymidine, or DL-phenylalanine, in filtered sea water. The treated animals were fixed in four changes of acetic-alcohol (1:3) of approximately 10 minutes each, at room temperature in the earlier experiments but at refrigerated temperature in later ones (the lower temperature decreased the amount of muscular contraction). The fixative was washed out in four changes of 70% ethanol; the last change was overnight at 4° C. The animals were then dehydrated and embedded in paraffin. Serial sections were cut at 5 μ. Control sections were extracted as follows: (1) worms treated with uridine-H^3: slides of tissue sections were extracted in ribonuclease (Worthington, 0.02% at pH 6.5 for one to two hours at 37° C.); (2) worms treated with thymidine-H^3: tissues sections were extracted in deoxyribonuclease (Worthington, 0.02% or 0.04% in 0.003 *M* $MgSO_4$ at pH 6.5 for one to two hours at 37° C.); (3) worms treated with DL-phenylalanine-H^3: (a) a few slides were placed in ribonuclease as in (1) above; another group was placed in deoxyribonuclease as in (2) above; (c) some tissue sections were digested in deoxyribonuclease as in (2) followed by ribonuclease as in (1); (d) other tissue sections were hydrolyzed in 5% TCA at 90° C. for 30 minutes to remove both DNA and RNA.

After the extractions, in making the first sets of radioautographs, both extracted sections and those not digested with enzymes were placed in dilute solutions of the appropriate non-labeled precursor (uridine, thymidine, or DL-phenylalanine) in distilled water and air-dried. For the rest of the radioautographs, extracted sections and those not digested with enzymes were treated for 5 minutes in 5% trichloracetic acid (TCA) at 2–4° C. to remove acid-soluble nucleotides. Sections were then passed through three changes (10 minutes each) of cold 70% ethanol and air-dried. Some of the sections of worms treated with thymidine-H^3 (including samples of sections digested in deoxyribonuclease and non-extracted sections) were hydrolyzed in 1 N HCl and stained with Feulgen; slides were subsequently air-dried from 70% ethanol.

The dried slides were then placed in distilled water in a water bath at 44–45° C. and coated with Kodak NTB3 liquid emulsion. After drying, slides were placed



FIGURES 1–4.

in sealed black boxes at 4° C. Following various exposure times, coated slides were developed in D-11 and air-dried.

The sections of budding worms which had been treated with thymidine-H^3 and stained with Feulgen were then either mounted directly with euparal or were first counterstained with 2% acidified fast green, and mounted in Canada balsam. The remaining sections of animals treated with thymidine-H^3, and most of the sections of animals treated with uridine-H^3, were stained with 0.025% azure B in pH 4 McIlvaine's buffer at 37° C. for periods up to 15 minutes. After staining with azure B (Woodard, Rasch and Swift, 1961) most of the slides were rinsed in distilled water for several minutes, air-dried (this avoided the white precipitate formed after tertiary butyl alcohol) and mounted in euparal. The rest of the sections of animals treated with uridine-H^3, and the majority of sections treated with DL-phenylalanine-H^3, were stained for 15 to 30 seconds in 0.1% toluidine blue at pH 6, rinsed in 95% ethanol, air-dried, and mounted in euparal.

The remainder of the sections of worms treated with DL-phenylalanine-H^3 were stained one hour in 1% aqueous fast green at pH 2 (Woodard, Rasch and Swift's modification, 1961, of Schrader and Leuchtenberger, 1950).

Results

In radioautographs, silver grains over a particular structure were interpreted as an indication of incorporation of the radioisotope precursor into that structure and as signifying synthesis; the presence of silver grains after short pulses may indicate initial synthesis, after longer pulses may indicate transfer or accumulation as well as synthesis.

RNA synthesis. In the developing oocytes of *Autolytus edwarsi,* incorporation of uridine-H^3, as evidenced by overlying silver grains, was noted primarily in cell structures which were shown cytochemically to contain RNA. Developing oocytes of stolons which had been treated with uridine-H^3 for two to fifteen minutes show incorporation of the radioisotope primarily over nucleoli (Fig. 1). In two stolons, some of the eggs after a 5-minute pulse and 10-minute pulse, respectively, had a few silver grains over the non-nucleolar portion of the nucleus although most of the grains were concentrated over the nucleolus. Other eggs of these two stolons

All figures are radioautographs of sectioned material which had been coated with Kodak NTB3; the photomicrographs were all taken at the same magnification with Contrast Process Ortho film.[2]

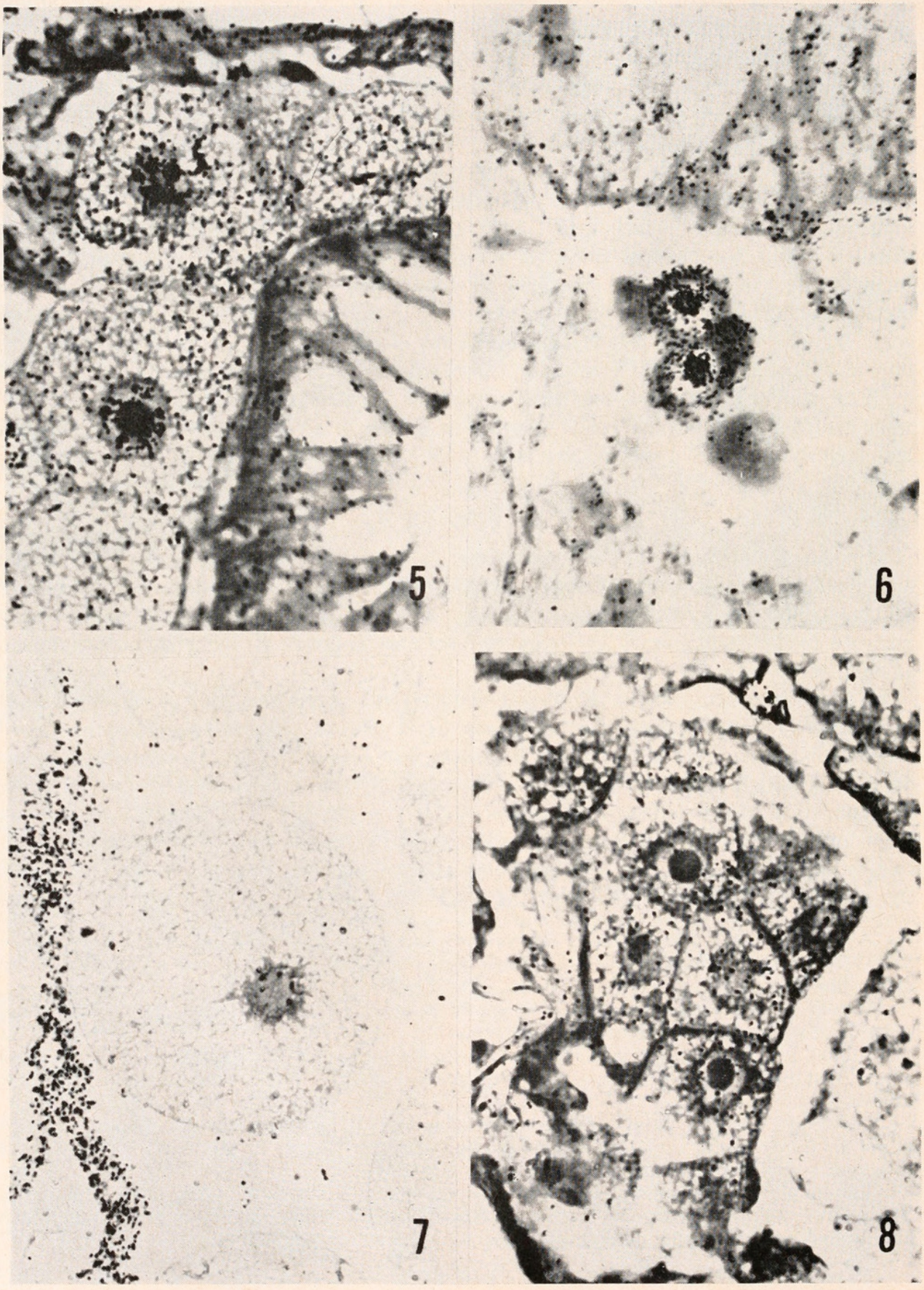
FIGURE 1. A large oocyte given a 10-minute pulse of uridine-H^3; exposure, 14 days; stain, azure B at pH 4. Silver grains over nucleolus only.

FIGURE 2. Two oocytes given a 30-minute pulse of uridine-H^3; exposure, 14 days; stain, azure B at pH 4. Silver grains over non-nucleolar regions of the nucleus as well as the nucleolus.

FIGURE 3. Oocytes given a 2-hour pulse of uridine-H^3; exposure, 3 days; stain, azure B at pH 4. Silver grains over the nucleoli and non-nucleolar regions of the nuclei, and beginning to appear over the cytoplasm.

FIGURE 4. RNase-digested control for oocytes shown in Figure 2; stain, azure B at pH 4. The arrows indicate the diameter of the nucleus. Note the absence of silver grains other than background, as well as the absence of color.

[2] The first three figures were published by the writer's permission in Dr. J. R. Collier's review article in "Current Topics in Developmental Biology," Vol. I, 1966, Academic Press, New York.



FIGURES 5–8.

showed no grains other than background over any part of the egg. Radioautographs of eggs exposed to uridine-H^3 for 30 minutes show grains over the nucleolus and over the rest of the nucleus (Fig. 2). In Figures 1 and 2 the eggs had the same exposure time so the number of grains indicates the relative amount of RNA synthesis. Oocytes exposed to the radioisotope for two hours show a few grains over the cytoplasm as well as over the nucleolus and non-nucleolar regions of the nucleus (Fig. 3). Further increase in length of exposure to uridine-H^3 resulted in an increased number of silver grains over the cytoplasm (Fig. 5), especially in the case of younger eggs (Fig. 6). This difference between older and younger eggs is even more marked than the photomicrographs suggest as the younger eggs received a shorter pulse (9 hours instead of 16 hours) and a shorter exposure period (3 days instead of 8 days) than did the older oocytes. In radioautographs of eggs pre-digested with RNase and stained with azure B, the color reaction for RNA was absent and very few silver grains were present (Fig. 4). These results were interpreted as showing that the most rapid synthesis of RNA occurs in the nucleolus of the developing oocytes of *A. edwarsi,* from which it migrates to the rest of the nucleus and then to the cytoplasm. The presence of the few grains over the non-nucleolar regions in some eggs after 5-minute and 10-minute pulses may indicate synthesis of chromosomal RNA or, as compared with other eggs, a precocious migration of nucleolar RNA into the nucleoplasm. Figures 3 and 5 indicate that only slight transfer of nuclear RNA has occurred within two hours and that only moderate transfer has been accomplished after 16 hours (some of the cytoplasmic grains in Figure 5 are background). After a 20-hour pulse, results are similar to those observed after a 16-hour pulse. Thus the nucleolus appears to be extremely active in RNA synthesis during the growth period of oogenesis, and migration of RNA to the cytoplasm is a relatively slow process, at least during the latter part of the growth period.

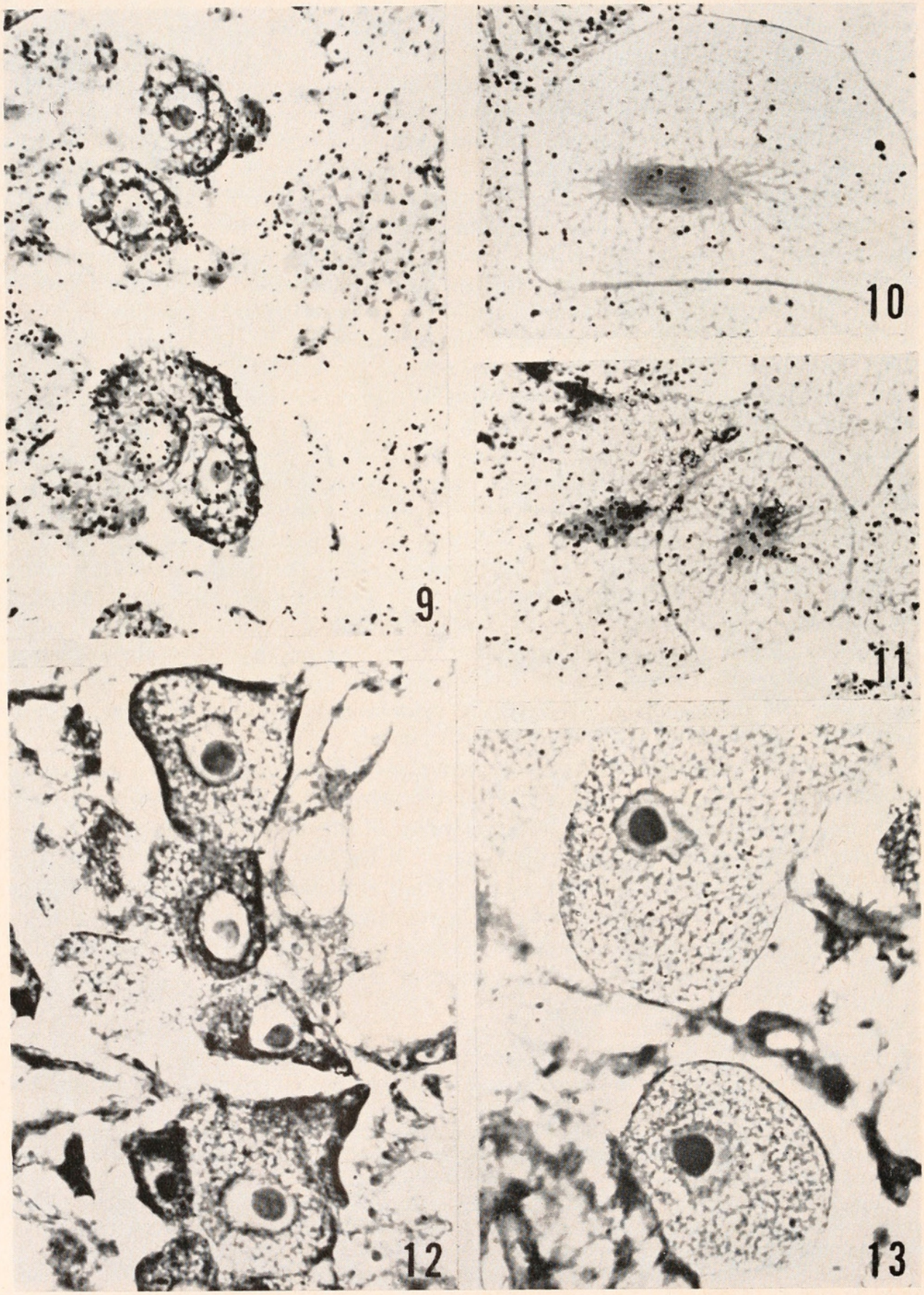
A sacconereis stage with the body cavity packed with fully grown oocytes in metaphase I (the eggs of *Autolytus* are fertilizable at this stage; Allen, 1964, and Gidholm, 1965) was given a 20-hour pulse of uridine-H^3. The results are illustrated by Figure 7. Of particular interest is the absence of grains (other than background) over all parts of the eggs. Thus, provided the permeability of the egg to uridine has not changed, it would appear that no significant RNA synthesis is occurring in fully grown eggs that are ready to be fertilized; this suggests that the synthetic machinery for RNA is turned off by the end of the growth period in this species.

FIGURE 5. Oocytes given a 16-hour pulse of uridine-H^3; exposure, 8 days; stain, azure B at pH 4. Silver grains over the nucleoli and the non-nucleolar regions of the nucleus, and more obvious over the cytoplasm than in Figure 3.

FIGURE 6. Two young oocytes given a 9-hour pulse of uridine-H^3; exposure 3 days; stain, azure B, pH 4. In comparison with Figure 5, the silver grains are more concentrated over the cytoplasm.

FIGURE 7. A fully grown oocyte at metaphase I (polar view showing 6 chromosomes) given a 20-hour pulse of uridine-H^3; exposure, 3 days; stain, azure B, pH 4. Note the absence of silver grains other than background.

FIGURE 8. Oocytes given a 30-minute pulse of phenylalanine-H^3; exposure, 21 days; stain, azure B, pH 4. Note that silver grains are scattered over nucleoli, non-nucleolar portions of nuclei, and cytoplasm.



FIGURES 9–12.

Protein synthesis. The number of budding worms treated with phenylalanine-H^3 was smaller than in the case of uridine-H^3, and very few of those treated had large oocytes. Following pulses of 30 minutes to 20 hours, radioautographs showed scattered silver grains over all parts of the developing eggs. Extraction with ribonuclease, deoxyribonuclease or hot TCA did not prevent fast green from staining the eggs, nor did it reduce the number of silver grains; therefore, it was assumed that phenylalanine-H^3 was being incorporated specifically into proteins. As shown in Figures 8 and 9, there are scattered grains over all parts of the developing eggs with no concentration of grains over the nucleolus. It thus seems apparent that all parts of developing oocytes (nucleoli, non-nucleolar regions of the nucleus, and cytoplasm) are synthesizing proteins, and that the nucleolus is no more active than any other part of the egg in this synthesis.

An older female stolon which had been given a 20-hour pulse was found to have the body cavity packed with a mixture of fully grown oocytes and cleavage stages (presumably fertilization had occurred accidentally, as normally in *Autolytus* fertilization and early development take place in a ventral egg sac or brood pouch; Gidholm, 1965, and Allen, 1964). Figures 10 and 11 are photomicrographs from the same radioautograph. Figure 10 shows an undivided egg in metaphase I; Figure 11 shows a cleavage stage in metaphase (some of the grains are background). A comparison of the two stages shows that the fully grown unfertilized egg is synthesizing little, if any, protein. In contrast, the grains over the cytoplasm of the cleaving blastomeres indicate that protein synthesis is occurring. Of particular interest, however, is the concentration of grains over the nuclear regions and spindles of cleaving blastomeres and the absence of grains other than background over the spindles of unfertilized eggs. Thus it seems clear that proteins for the mitotic spindles are being synthesized during cleavage whereas the proteins for the meiotic spindles are synthesized prior to the maturation divisions.

DNA synthesis. Budding female stocks at various stages of differentiation were treated with thymidine-H^3 following pulses of five minutes to twenty hours; radioautographs of the stolons showed no silver grains other than background over the eggs. Figures 12 and 13 show radioautographs of developing oocytes following a 60-minute pulse. The absence of silver grains indicates that the oocytes of *A. edwarsi,* whether they are large or small, do not incorporate thymidine-H^3. This is not because this precursor of DNA was not diffusing to them, for aggregates of silver grains (indicating incorporation and hence synthesis of DNA) were observed over nuclei in parapodia and other tissues in the same radioautographs.

FIGURE 9. Young oocytes given a 60-minute pulse of phenylalanine-H^3; exposure, 60 days; stain, toluidine blue at pH 6. Even with a longer pulse and longer exposure period than the eggs shown in Figure 8, silver grains are still not concentrated, but are scattered over the entire oocyte.

FIGURES 10 AND 11. A fully grown unfertilized oocyte (Fig. 10) and a cleavage stage (Fig. 11) from the same radioautograph; both were simultaneously given a 20-hour pulse of phenylalanine-H^3; exposure 6 days; stain, azure B, pH 4; some of the silver grains are background. In comparison with the younger oocytes in Figures 8 and 9, note the scarcity of grains over the fully grown oocyte. In comparison with the cleavage stage (Fig. 11), note the absence of silver grains other than background over the meiotic spindle, and the few grains in the cytoplasm.

FIGURES 12 AND 13. Oocytes in different stages of growth, all from the same stolon, which were given a 60-minute pulse of thymidine-H^3; exposure 7 days; stain, azure B, pH 4. Note the absence of silver grains over all parts of the eggs, whether the oocytes are large or small.

That no significant synthesis of DNA was occurring in these oocytes is borne out by the staining reactions. DNA was demonstrated by Feulgen and azure B in various tissues of the stocks and differentiating stolons but not in developing eggs. Apparently this nucleic acid is too dispersed in developing oocytes of this polychaete to give a positive reaction with the cytochemical methods used. Earlier, this was found to be the case in fully grown oocytes of *Diopatra* (another polychaete); the writer, however, successfully demonstrated the presence of DNA by staining with gallocyanin-chromalum (Allen, 1961). In the case of *Autolytus* eggs, tests for DNA were negative following gallocyanin-chromalum. Judging, then, from radioautographs and cytochemical tests, the DNA in the germinal vesicle of *Autolytus edwarsi* is diffusely distributed and presumably was synthesized very early in oogenesis.

Discussion

The preferential uptake of uridine into the nucleolus of *Autolytus* oocytes is in essential agreement with the results from other eggs (Tweedell, 1966; Piatigorsky *et al.*, 1967). RNA synthesis is more rapid during the earlier part of oogenesis, while there is no significant synthesis of RNA in the fully grown egg; however, the absence of information concerning uptake of uridine by the unfertilized egg prevents one from drawing a definite conclusion (compare, for example, Piatigorsky and Whiteley, 1965; Gould, 1965; Siekevitz *et al.*, 1966). Thus at the end, or near the end, of the growth period, genetic transcription may cease in *Autolytus*.

The most rapid synthesis of RNA in *Autolytus* oocytes occurs in the nucleolus from which it migrates to the rest of the nucleus and then to the cytoplasm. Recently, Penman *et al.* (1966) have demonstrated in HeLa cells that all of the 45S precursor to ribosomal RNA is contained in the nucleolus and that this precursor yields a 16S RNA that is rapidly transferred to the cytoplasm, as well as a 35S fragment that remains in the nucleus for a short time. This 35S portion subsequently fragments to produce 28S ribosomal RNA that moves into the cytoplasm. The RNA in *Autolytus* oocytes which accumulates in the nucleolus and moves from there to the rest of the nucleus and then to the cytoplasm fits the pattern of transfer of ribosomal RNA in HeLa cells. Thus the RNA synthesized in *Autolytus* eggs may represent different types of ribosomal RNA. There is evidence that other oocytes (particularly their nucleoli) synthesize, or accumulate, relatively large amounts of ribosomal RNA (Davidson *et al.*, 1964; Brown, 1964, 1966; Brown and Littna, 1964b, 1966; Gross *et al.*, 1965; Vincent *et al.*, 1966).

From other work, it is known that messenger RNA is also produced in developing oocytes (Slater and Spiegelman, 1966; Davidson *et al.*, 1966; Crippa *et al.*, 1967), and probably transfer RNA (Vincent *et al.*, 1966). Most radioautographic studies, including the present one on *Autolytus*, provide insufficient evidence for determining whether or not the RNA that is rapidly synthesized in developing oocytes represents one or more types of RNA; however, the fact that protein synthesis is also occurring in *Autolytus* oocytes indicates that messenger RNA is produced during oogenesis.

The developing oocytes of *Autolytus* synthesize their own RNA during the growth period of oogenesis instead of relying on nurse cells. This is in contrast to the developing eggs of the polychaete, *Diopatra*, in which cytochemical evidence

suggests that RNA is passed from nurse cells across a cytoplasmic bridge to the developing oocyte (Allen, 1961). It is also in contrast to the developing eggs of certain insects which apparently are furnished at least part of their RNA by follicle cells, or by specialized nurse cells (Sirlin and Jacob, 1960; Favard-Séréno and Durand, 1963a; Vanderberg, 1963).

Favard-Séréno and Durand (1963a) observed that in the cricket ovary it is the chromosomes of the follicle cells that are the primary site of RNA synthesis. From its source RNA then migrates to the nucleolus where it accumulates before moving to the cytoplasm. It was pointed out in describing radioautographic results for *Autolytus* that the eggs of a few stolons are exceptions in having silver grains over the non-nucleolar regions of the nucleus after a pulse of 5 to 10 minutes. Differences in permeability of stolons of different ages might account for the variation or, as compared with the majority of eggs, the exceptions may indicate a precocious migration of nucleolar RNA into the nucleoplasm due to a variation in synthetic rates among nucleoli. Another possible interpretation is that RNA is being synthesized by the chromosomes and is migrating in the reverse direction, *i.e.*, from chromosomes to nucleolus, as in the follicle cells of the cricket ovary. Should this be the case, one must still assume that the nucleolus in these oocytes represents an extremely active site of RNA synthesis; otherwise, uridine would not be incorporated so rapidly into the nucleoli of the majority of oocytes. It is also possible that transfer in both directions is occurring; *i.e.*, that some grains over the non-nucleolar portions of the nucleus may indicate RNA migrating from chromosomes to the nucleolus, while others may represent RNA migrating from the nucleolus to the cytoplasm. The results of the present study suggest that the major movement would be the latter.

Protein synthesis. The evidence provided by the present investigation indicates that all parts of the developing oocytes of *A. edwarsi*—*i.e.*, nucleolus, the rest of the nucleus, and cytoplasm—synthesize proteins. There was no preferential localization of silver grains; in other words, the nucleolus did not appear to be a major source of synthesis (or accumulation) of proteins.

In contrast to the non-localized uptake of protein precursors in *Autolytus* oocytes, Ficq (1953, 1955a, 1955b) demonstrated that the nucleolus of starfish and amphibian oocytes is more active than the rest of the egg in incorporating glycine and phenylalanine, suggesting a higher degree of protein synthesis in the nucleolus. Later work on amphibian oocytes demonstrated that phenylalanine was incorporated uniformly in the cytoplasm, and also labeled the nucleus (Brachet and Ficq, 1956; Ficq *et al.*, 1958). Pantelouris (1958), using *Triturus* oocytes, demonstrated that the site of uptake of protein precursors and the direction of their migration within developing oocytes depends on whether or not vitellogenesis is occurring. Ficq, Pavan and Brachet (1958) had concluded that the nucleus, particularly the lampbrush chromosomes, may be active in synthesizing proteins. More recently, Gall (1963) demonstrated in oocytes of the newt that the lampbrush chromosomes incorporate phenylalanine throughout the length of their loops, thus providing evidence that these chromosomes are active in protein synthesis.

Brachet (1960) points out that radioautographic studies indicate that the nucleolus play a more conspicuous role in RNA synthesis than in protein synthesis. One can conclude from the radioautographs of *Autolytus* that RNA is being synthesized

more rapidly than proteins in the nucleoli of developing eggs. It has been demonstrated for the clawed toad that a marked synthesis of ribosomal RNA occurs during oogenesis (Brown, 1964), and that new ribosomal RNA does not occur until the beginning of gastrulation (Brown and Littna, 1964a). This suggests that the ribosomal RNA stored during oogenesis is the only ribosomal RNA available for protein synthesis up to the time of gastrulation. Perhaps the transfer of rapidly synthesized RNA from nucleolus to cytoplasm in *Autolytus* oocytes means that messenger and/or ribosomal RNA is being stored during oogenesis for later use, *i.e.,* for early embryonic development.

The radioautographic observations on *Autolytus* eggs indicate that no significant incorporation of DL-phenylalanine-H^3 is occurring in fully grown oocytes in metaphase I, while during cleavage incorporation does occur, being heaviest over the nuclear and spindle regions. A preferential incorporation of DL-leucine-H^3 into mitotic spindles of early cleavage stages has been demonstrated in the sea urchin (Gross and Cousineau, 1963; Gross, 1964), the label being highly selective for the spindles and nuclei. The evidence from radioautographs of *Autolytus* suggests that in these eggs also the proteins for the mitotic spindles are being synthesized during cleavage. In contrast, it would appear that proteins for the meiotic spindles are synthesized prior to the maturation divisions so presumably they are simply assembled during meiosis. It is well established that fully grown unfertilized eggs of the sea urchin show little metabolic activity but that a marked protein synthesis begins almost immediately after fertilization (Hultin, 1950, 1961; Giudice, *et al.,* 1962; Tyler, 1963; Gross, 1964); some of these new proteins are thus required for mitosis (Hultin, 1961; Gross, 1964).

DNA synthesis. As noted above, the evidence presented indicates that the developing oocytes of *A. edwarsi* do not incorporate thymidine-H^3. It thus seems evident that no significant DNA synthesis is occurring during the growth period of oogenesis, either in the developing germinal vesicle or in the cytoplasm. It was concluded, therefore, that nuclear DNA synthesis must occur very early in oogenesis. For another polychaete, *Pectinaria,* Tweedell (1966) recently demonstrated that nuclear uptake of thymidine-H^3 is restricted to the early period of oogenesis, *i.e.,* before the growth of the oocyte begins. Similarly, in various other species studied, the evidence shows that thymidine-H^3 is not incorporated into the nucleus during the growth period of the egg. This is true for oocytes of the sea urchin (Ficq *et al.,* 1963), the cricket (Favard-Séréno and Durand, 1963b), and the mouse (Mintz, 1964). Nor is thymidine incorporated into the germinal vesicle of the fully grown oocyte in most species studied (Ficq, 1961; Simmel and Karnofsky, 1961; Nigon and Nonnenmacher, 1961; Favard-Séréno and Durand, 1963b; Ficq *et al.,* 1963). Probably in all these forms synthesis of nuclear DNA occurs early in oogenesis as has been demonstrated for the mouse egg (Rudkin and Griech, 1962). Recently, in the adult promsimian, *Galago demidoffi,* Ioannou (1967) demonstrated incorporation of thymidine-H^3 by oocytes at pre-leptotene or leptotene as well as by oogonia in prophase. These developing germ cells of the adult ovary thus are capable of synthesizing DNA but whether they ever become functional eggs has not been determined.

In *Autolytus* eggs no cytoplasmic synthesis of DNA was evident. Tweedell (1966), however, was able to demonstrate cytoplasmic uptake of thymidine-H^3 in

developing *Pectinaria* oocytes, suggesting the synthesis of cytoplasmic DNA in these eggs. Pikó, Tyler and Vinograd (1967) recently have demonstrated DNA in mitochondria and yolk spheres of sea urchin eggs. They cite a number of papers that demonstrate that large amounts of cytoplasmic DNA occur in the mature eggs of many species of animals. For some oocytes, it has been shown that the associated follicle cells contribute to ooplasmic DNA. Incorporation of thymidine occurs in the nuclei of follicle cells in the cricket with a subsequent transfer of radioactive DNA into their cytoplasm and into the ooplasm (Favard-Séréno and Durand, 1963b). The possibility still exists that cytoplasmic DNA occurs in *Autolytus* oocytes but since no incorporation of thymidine was evident, it appears that no DNA synthesis was taking place during oogenesis.

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Summary

1. The incorporation of tritiated uridine, thymidine, and phenylalanine into nucleic acids and proteins was studied in the developing oocytes of stolons produced by asexual budding in *Autolytus edwarsi.*

2. Extremely rapid incorporation of uridine-H^3 into the nucleolus of developing oocytes suggests the importance of the nucleolus in the synthesis of RNA. Oocytes exposed to uridine for successively longer periods than two to fifteen minutes show silver grains over the non-nucleolar regions of the nucleus as well as the nucleolus, and finally a number of grains appear over the cytoplasm (the relative number of cytoplasmic grains is greater in younger oocytes). Results indicate that RNA is synthesized mainly in the nucleolus of developing oocytes from which it migrates to non-nucleolar regions of the nucleus and then to the cytoplasm. Transfer of RNA to the cytoplasm is a relatively slow process in these eggs.

3. Fully grown oocytes at metaphase I (the stage at which *Autolytus* eggs are fertilizable) do not incorporate uridine-H^3, indicating that no significant synthesis of RNA is occurring at the close of the growth period of oogenesis.

4. Phenylalanine-H^3 was incorporated into all parts of the developing oocytes, and was not incorporated more rapidly into the nucleolus. Thus it seems apparent that all parts of developing eggs (nucleoli, non-nucleolar regions of the nucleus, and cytoplasm) are synthesizing protein. Fully grown oocytes in metaphase I, however, are synthesizing little, if any, protein. In contrast to ripe eggs, cleavage stages are synthesizing proteins, particularly in nuclear and spindle regions. The results for cleavage stages are similar to those of the sea urchin. The results for *Autolytus* suggest that in these eggs the proteins for the mitotic spindles are being synthesized during cleavage whereas the proteins for the meiotic spindles are synthesized prior to the maturation divisions.

5. Thymidine-H^3 was not incorporated into developing oocytes, indicating that no significant DNA synthesis was occurring. Radioautographs and cytochemical tests indicate that DNA is diffusely distributed in the germinal vesicle of *Autolytus edwarsi.* Presumably in this species, DNA is synthesized very early in oogenesis.

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