THE INCORPORATION OF ^{14}C-ADENINE INTO THE OOCYTES OF *ASELLUS AQUATICUS* AS STUDIED BY AUTORADIOGRAPHY

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Received March 31, 1960

In a variety of cells, the nucleolus appears to be a centre of intensive metabolism. Repeatedly, the incorporation of phosphorus, amino acids and adenine or cytidine as precursors for proteins and RNA has been shown to be markedly higher in the nucleolus than in the nucleus at large or in the cytoplasm [7, 9, 11, 12, 25, 27, 28, 29, 32]. The synthesis or turnover of RNA thus demonstrated is thought to be connected with protein synthesis in or on behalf of the cytoplasm, but only in some cases it has been possible to tentatively relate nucleolar activity to any specific cytoplasmic process [8, 9].

At a time when tritiated precursors were not yet available for the experiments, *Asellus aquaticus* was chosen as a test animal because both the oocytes and the cells of the intestinal glands have nucleoli amenable to autoradiographic investigation with ^{14}C [24]. Moreover, it has been reported [1, 6] that the cycle of oviposition in this animal is closely linked to the moulting periods, so that it seemed possible to obtain oocytes of known grades of development by selecting animals at various stages of the moulting cycle. Such stages could conceivably be determined from external characteristics such as the size of the breeding pouch lamellae [6, 17].

MATERIALS AND METHODS

Test animal.—The ubiquitous freshwater isopod *Asellus aquaticus* L. was caught in ditches around Utrecht and could be kept alive for months in freshwater aquaria with a vegetation mainly consisting of *Elodea canadensis* and some dead leaves added for food [6].

During the months of July through December, a total of 237 females were isolated in glass dishes and observed daily to study the moulting cycle. It was found that most animals moult 2–3 days after having been transferred either from the aquarium or from their natural environment, even when the type of water and vegetation was

carefully kept alike before and after the transportation. This moulting is therefore thought to be a reaction to the manipulation or to the imposed confinement; it may interfere with the natural moulting cycle.

After this first "reactive" moulting, the majority of animals were seen to moult a varying number of times, often at very unequal intervals (at the shortest 2 days, at the longest 40 days, median value 7–8 days). Even in one and the same animal the variation sometimes was so large that hardly any regularity in the moulting cycle could be observed. Such animals were discarded.

From those animals which did show a fairly regular succession of moults, a number were killed at respectively 0, 4, 8–9, 11–12 and 16–17 days after the last moult. Of each of these animals were measured: (1) length of the breeding pouch lamellae (epipodites [6, 17]) in relation to thorax width; (2) the mean surface areas of cytoplasm, nucleus and nucleolus from equatorially sectioned oocytes. The relation between oocyte development and moulting cycle was then studied from these measurements (see below).

When it was found that all animals thus selected contained oocytes in the earlier stages of development, these were combined into a *first experiment* and a *second experiment* was done with females taken directly from the aquarium as they were found coupled to a male. The latter group of animals all provided oocytes in the later stages of development, i.e. approaching maturity.

Isotope injection and histological procedures.—All animals were injected with 0.002 ml saline as prescribed by Needham [18], in which was dissolved 0.04 μC 8-^{14}C-adenine sulphate hemihydrate (from Radiochemical Centre, Amersham, U.K.), containing 1.06 mC/m*M* as measured by a thin-window Geiger counter.

Before injection the animals were lightly anaesthetized by putting them in 50 ml water over 3 ml chloroform on a wad of cotton-wool to prevent direct contact with the narcotic. The immobile animal was stretched and fastened under a dissecting microscope and the 3rd or 4th thoracic limb was clipped off at the coxa. A thin hollow glass needle with an obliquely ground tip was then introduced through the coxa stump well into the median section of the thorax. The glass needle was connected to an Agla Micrometer Syrinx filled with water. A drop of Sudan III-stained petroleum–ether was inserted between the water and the injection fluid, so that the glass needle only contained an amount of the latter sufficient for 5 to 6 successive injections. Injection through the amputated leg seemed favourable as autotomy of limbs is a regular occurrence in *Asellus* and the wound is closed very soon [17].

Immediately after injection the animals were placed in tap water; the narcotic was seen to work off in 10–15 minutes. The animals were killed invariably 3 hours after injection, fixed in ethanol–acetic acid 3:1 for 1 hour and embedded in paraffin through the usual histological procedures. Seven μ sections were mounted on glass slides previously coated with gelatin. Of any one animal, successive sections nos. 1 ... 6, 7 ... 12 etc. were arranged on six slides so as to obtain 6 nearly identical preparations. These were dewaxed, placed in $\frac{1}{2}$ per cent non-labeled adenine for 15 minutes, washed, placed in Lugol solution for 2 hours to reduce the often excessive chemography caused by the pyloric caeca, washed for 24 hours in running tap-water and dried in a cold air stream. One slide of each set of six was placed in 10 per cent perchloric acid at 4°C during 24 hours under continuous gentle shaking to remove RNA [12], one in 5 per cent perchloric acid at 60°C during 1 hour to remove both

RNA and DNA. For autoradiography, all slides were coated with one and the same batch of Ilford G5 emulsion in gel form.

Autoradiographic technique.—The coating method as described by Ficq [9, 10] was used with slight modifications. It was found convenient to draw the molten emulsion from the bottom of a glass tube by means of a pipette instead of pouring it from a beaker. The glass tube was kept in a Dewar flask with water of 48–50°C; the pipette was so constructed that, by applying finger pressure, a predetermined quantity of liquid emulsion was drawn to match the area of slide to be covered with emulsion at a desired thickness, the emulsion being subsequently poured onto the slide by releasing the pressure. As the emulsion was drawn from the bottom of the tube, it was not necessary to pass it through a gauze to remove air bubbles, which in turn resulted in a more economical use of the emulsion.

The preparations were exposed from 5 to 35 days at approx. 4°C, developed in Amidol developer after Dilworth *et al.* [5] for 20 minutes at 14°C, washed in distilled water for 30 seconds, fixed in 1/3 sat. hyposulphite for 3 minutes at 14°C, washed in running tap-water for 1 hr, stained with methyl green-pyronine (Unna) and mounted in Cedax.

A number of oocytes in various stages of development were selected and the number of β-tracks from ^{14}C was counted separately over nucleolus, nucleus and cytoplasm, a β-track being recognized as such when it showed at least three grains in an interrelated position. The density of loose-grain background was such that a chance juxtaposition of background grains to be mistaken for a β-track was deemed very small: about one in 800–5000 tracks.

Background of β-tracks was counted in at least 10 areas of 100 μ^2 around and between and always at > 100 μ distance from the respective sections. Surface areas of nuclei and cytoplasm were determined by planimeter readings on camera lucida drawings; the surface areas of nucleoli facing the photographic emulsion were calculated from the diameters as measured by a calibrated eye-piece micrometer.

After subtraction of background counts, all values were calculated per unit surface area and exposure time so as to obtain arbitrary but comparable figures.

Determination of oocyte stages.—As said before, it was unexpectedly found that all animals of the *first experiment* (see p. 202) presented oocytes in the earlier stages of development. A perusal of the measurements described earlier showed that under the given conditions neither the number of days after the last moult nor the size of the breeding pouch lamellae can with sufficient certainty be used as an indication of the stage of the oocytes.

Therefore these stages had to be assessed mainly by other criteria. As such were chosen: (1) mean diameter of oocytes, (2) same of nucleus, (3) same of nucleolus, (4) estimated quantity of vacuoles in cytoplasm, (5) length of breeding pouch lamellae. Rank numbers were allotted to every animal for each of these criteria in increasing order.

In the *second experiment*, all oocytes were approaching maturity. In these phases, increase in size is no longer indicative of the stage, the final girth

of the oocyte being mainly determined by the size and nutritional condition of the animal. There are minor changes in the yolk vacuoles, but the prominent feature of these stages is the shrinking of the nucleus and the disappearance of the nucleolus. The latter phenomenon was determined more precisely

TABLE I. *Some characteristics of chosen oocyte stages*

Animal No.	Code No.	Days after moult	Breeding pouch lamellae[a]	Stage	Mean diameter of oocyte	Mean diameter of nucleus	Mean diameter of nucleolus	Percentage of sections showing nucleolus: whole	Percentage of sections showing nucleolus: partly dissolved
First experiment									
1	8513	—	—	A	40	27	11	—	—
2	42	12	1/2	B	62	30	13	—	—
3	22	4	1/2	B	68	30	13	—	—
4	43	11	4/5	C	78	39	13	—	—
5	11	0	1/1	C	79	36	15	—	—
Second experiment									
6	75	—	>1/1	D	133	Irregular	16	7 (175 in 2490)	0.9
7	85	—	>1/1	E	123	Irregular	16	5.4 (75 in 1328)	0.5
8	78	—	>1/1	F	240	Irregular	25	4.7 (45 in 942)	0.5
9	83	—	>1/1	F	220	Irregular	17	0.9 (3 in 336)	—
10	25	—	>1/1	G	206	Irregular	—	None (0 in 1209)	—

[a] In relation to half thorax width.

by assessing, for each animal, the percentage of oocyte sections in which a nucleolus was seen. As the diameters of the oocytes vary largely between animals and those of the nucleoli comparatively little, a larger oocyte will yield a greater number of sections without a nucleolus than will a smaller oocyte and therefore the percentages found have been corrected for the mean diameter of the oocytes. Thereupon, rank numbers were allotted for each of the following characteristics: (1) appearance of cytoplasm (maturity of yolk granules), (2) appearance of nucleus (in order of increasing shrinkage),

(3) percentage (corrected) of oocyte sections showing a nucleolus, in decreasing order, (4) percentage of nucleoli partly vanished.

For both experiments, the rank numbers allotted to any one animal were added up and the nine animals suitable for further study were then arranged according to the totals thus obtained. One animal of an earlier experiment by the first author, which had oocytes of a much younger stage, was included in this study. All relevant data are combined in Table I.

RESULTS

Assessment of the stages of oocyte development covered.—The development of the oocytes of *Asellus aquaticus* does not differ essentially from that of many other invertebrates [31]. For convenience, the process is divided into four periods. Within each period, the stages of development found in the animals selected for this study are thereupon assessed; for the earlier stages the judgment was partly guided by the detailed histological study by Montefoschi and Magaldi [16]. The ten animals selected were judged to represent seven stages, hereafter designated A through G (see Table I), because in some cases two animals of essentially the same stage were grouped together. A survey of the four periods together with the stages found in these experiments is presented in Table II. A detailed description is given below.

1. The *first period* begins with the development of the oocytes out of the oogonia. In *Asellus*, the young oocytes measure 7–15 μ, the nucleus is surrounded by only a thin layer of cytoplasm. The principal changes during this period take place in the nucleus, which passes through the prophase of meiosis. Pre-leptotene, leptotene, zygotene and pachytene stages have

TABLE II. *Stages of oocyte development studied*

Period	Main feature	Stage	Represented by animal No.
1st	Meiosis	—	None
2nd	Cell growth	A	1. Early in 2nd period
		B	2 & 3. Transition to 3rd period
3rd	Yolk accumulation	C	4 & 5. Beginning of 3rd period
		D	6. End of 3rd period
4th	Maturation	E	7. First signs of maturation
		F	8 & 9. Middle of 4th period
		G	10. Mature egg

been identified [16] and it is supposed that at the latter stage meiosis is interrupted, to continue only after the oocyte has passed through the entire growth and yolk formation phases [31].

Oocytes of the first period were not included in the present study.

2. The *second period* is characterized by a slow growth of the oocytes (diameter increase from 15 to 60–80 μ), in which nucleus and cytoplasm grow at approximately equal rates. A complex of changes has been observed [16], which strongly points towards a displacement of nucleolar and nuclear material to the cytoplasm. It is supposed that these changes ultimately lead to the beginning of vitellogenesis, which marks the end of this period.

The *nucleolus* is at first homogeneous and basophilic and exhibits a strong alkaline phosphatase reaction [23], such as is common to nucleoli [4, 30]. Later on it shows mostly one acidophilic vacuole. Such vacuolization has been observed in the oocyte nucleoli of many species at this stage of development [30]. A connection between nucleolar changes and vitellogenesis has likewise been indicated for a number of species [14, 30].

In the *nucleus*, an acidophilic, slightly Feulgen-positive mass, called "semilunar structure" [16] accumulates at one side along the nuclear membrane. In later stages, the nucleolus is often seen in close contact with this structure and it has been suggested that the contents of the nucleolar vacuoles are emptied into it [16].

The *cytoplasm* stains increasingly with pyronine and most prominently so near that side of the nucleus where the "semilunar structure" is attached to the nuclear membrane. Shortly after the cytoplasm has become pyroninophilic all over, the first small vacuoles appear in it, which mark the beginning of yolk formation.

At the same time, the *follicle cells* adhering to the outside of the oocyte have multiplied to such an extent, that they now form an uninterrupted layer around the egg cell (Fig. 1).

In this investigation, two stages were found to fall into this period:

Stage A

Early half of second period. Represented by animal no. 1.

No. 1. The cytoplasm is homogeneously strongly pyroninophilic and contains no vacuoles. The nucleus is comparatively large, oval, vesicular and sharply outlined. The nucleolus is very dense and stains intensely with pyronine.

Stage B

Transition from second to third period, marked by the appearance of yolk vacuoles. Represented by animals nos. 2 and 3.

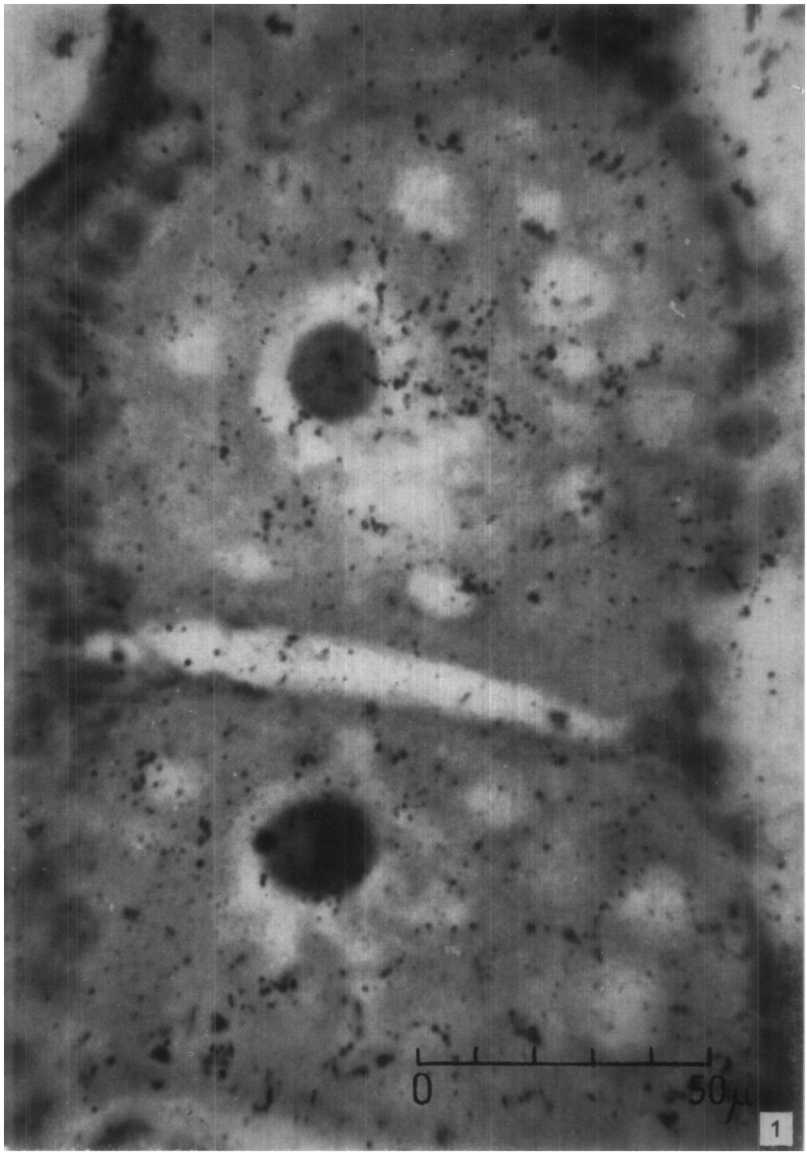

Fig. 1.—Young oocytes of *Asellus aquaticus* (Stage C as defined in text) shortly after the onset of yolk formation. Unna stain, coated autoradiograph.

No. 2. In some oocytes the cytoplasm contains a few non-pyrononophilic vacuoles which mark the beginning of yolk formation. The nucleus is oval, sharply outlined and shows a local accumulation of chromatin on the nuclear membrane which corresponds with the "semilunar structure" [16]. The nucleolus is intensely pyroninophilic and may contain a lighter area or vacuole.

No. 3. The cytoplasm generally contains somewhat more vacuoles than in No. 2, but there are still some oocytes without. The nucleus sometimes shows the "semilunar structure" more clearly than in No. 2 and the nucleolus is often seen in close association with it.

3. The *third period* is generally characterized by a rapid increase in size of the oocyte (diameter increase from 60–80 to 120–200 μ), which is now mainly brought about by the accumulation of yolk. When the first signs of yolk formation appear as small vacuoles (Fig. 1), the *cytoplasm* itself becomes less and less pyroninophilic. The impression was gained that the amount of cytoplasm proper does not increase in the whole of this period and that growth is entirely effected by the increase in size and number of yolk vacuoles or granules (Fig. 2). The mean surface of 40 equatorially sectioned oocytes of stage B (see above) was measured and found to be 901 units, of which no more than 4 per cent was taken up by yolk vacuoles. The mean surface of 50 equatorially sectioned oocytes of stage D (see below) was 5422 units. Parts of various oocytes were photographed and the areas occupied by the yolk granules and cytoplasm images respectively cut out and weighed: these parts were found to constitute respectively 81.5 and 18.5 per cent of the total area. The cytoplasm surface of a section at stage D would then be 18.5 per cent of 5422 = 1003 units, which indeed does not differ a great deal from the 901 — 4 per cent = 865 units for stage B. Thus it may be inferred that also the total volume of cytoplasm proper does not increase very much during the whole of the third period.

In this period, the *follicle cells*, which surround the oocyte, are intensely pyroninophilic and show a marked incorporation of ^{14}C-adenine (Fig. 2). A similar activity has been observed in the follicle cells of *Rana fusca* oocytes after administration of ^{14}C-glycine [8]. It seems logical to suppose that their activity is related to the accumulation of yolk, but their role has not been investigated in detail.

The *nucleus* shows little increase in diameter and there are no striking changes in its appearance. The *nucleolus* increases in diameter from 15 to 18–22 μ and remains strongly basophilic with some acidophilic vacuoles throughout this period.

In the material studied, the period was found to be represented by the two widely different stages C and D. The most advanced oocytes from the first

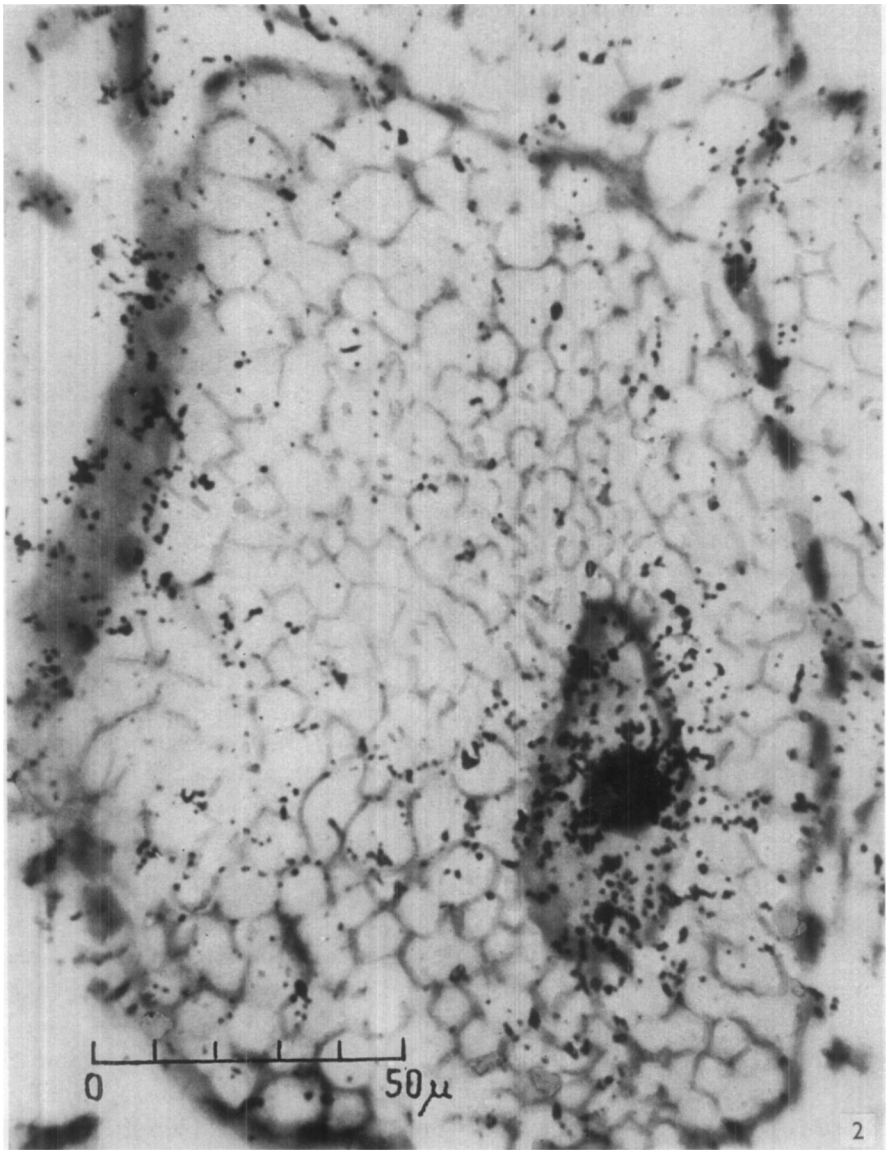

Fig. 2.—Almost mature oocyte of *Asellus aquaticus* (Stage D as defined in text). Unna stain-coated autoradiograph.

experiment (see p. 202) clearly belonged to the beginning of this period (stage C), the least advanced ones of the second experiment represented its final stage (D). Very probably there is a considerable time lapse between stages C and D, but as there is merely a continuous growth by yolk accumulation without any essential cytophysiological change between these two, it was not endeavoured to close the gap by adducing intermediate stages. Hence:

Stage C

Beginning of third period. Represented by animals nos. 4 and 5.

No. 4. The cytoplasm always contains vacuoles and there are many more of them than in No. 3. Nucleus and nucleolus have almost the same appearance as in the earlier numbers, but the nuclear membrane may be a bit less distinct where it is closest to the nucleolus (Fig. 1).

No. 5. The cytoplasm is largely filled with vacuoles. In the nucleus, the chromatin forms fine threads which lay evenly distributed throughout the nuclear space. The distinctness of the nuclear membrane varies. The nucleolus is as strongly pyroninophilic as before and sometimes shows a darker sickle at its periphery.

Stage D

End of third period. Represented by animal No. 6.

No. 6. The cytoplasm is entirely filled with comparatively small yolk granules; all spaces in between are taken up by granular cytoplasm. The nucleus is of irregular shape but clearly outlined. Nucleoli seldom show the signs usually preceding their disappearance (see below) (Fig. 2).

4. The fourth period represents the final maturation of the oocytes. Growth by yolk accumulation has now practically come to a standstill; the cytoplasm is always completely filled with opaque yolk granules. The nucleus shrinks as its histologically "empty" spaces disappear; in the later stages it loses its rounded shape and finally it is seen as an irregularly shaped shrunken mass of approximately the same optical density as the adjacent cytoplasm. The nucleolus is less densely pyroninophilic than in the earlier stages and often shows a large vacuole. The outer pyroninophilic shell fades and finally the nucleolus disappears altogether. Both the appearance of acidophilic vacuoles and the subsequent disappearance of nucleoli just before the oocyte reaches full maturity are of common occurrence [2, 14, 30].

These changes are believed to be accomplished in a comparatively short time in *Asellus*. Stages E, F and G, located in this period, as well as stage D at the end of the preceding one, succeed each other at rather shorter time intervals than the stages described earlier.

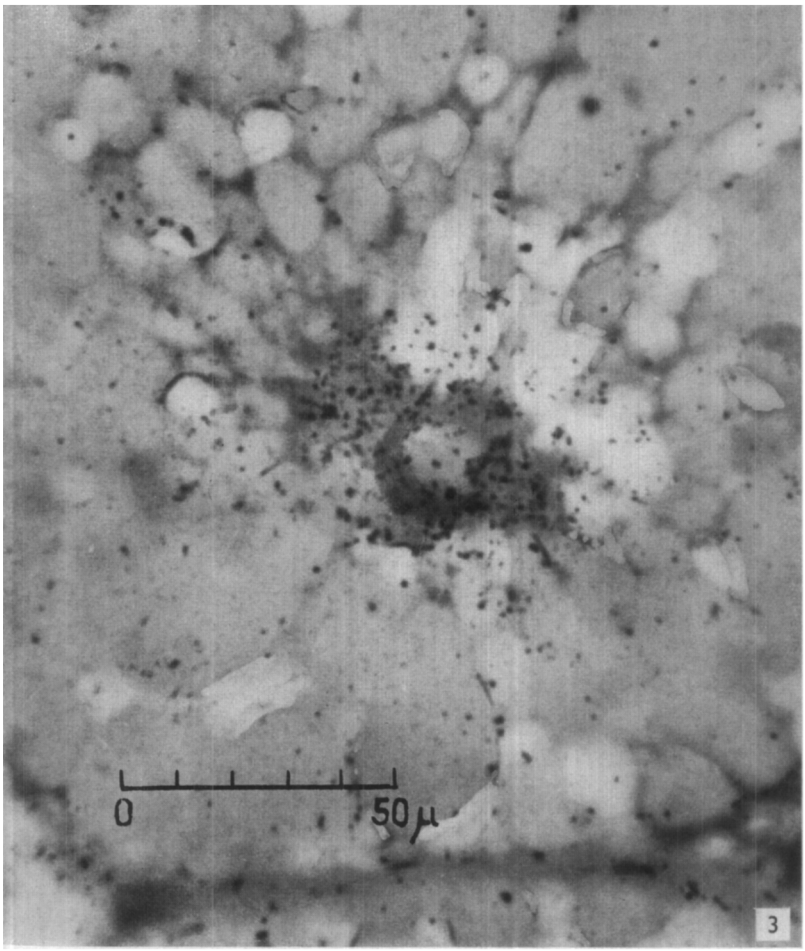

Fig. 3.—Part of mature oocyte of *Asellus aquaticus* (Stage F as defined in text). The nucleolus, which is now disappearing, shows the middle part devoid of pyronine affinity and of adenine incorporation. Unna stain, coated autoradiograph.

Stage E

Beginning of fourth period: first indication of maturation changes. Represented by animal No. 7.

No. 7. The cytoplasm closely resembles that of No. 6, but the nucleus is slightly less clearly outlined and of a less regular shape. Nucleoli are approximately as in No. 6.

Stage F

Middle of fourth period: nucleolus in process of dissolution. Represented by animals Nos. 8 and 9, which are not fully identical as to their maturation stages but are taken together for statistical evaluation.

No. 8. The yolk granules are somewhat larger but less distinct than in the other animals of this experiment. The overall appearance of the oocytes places them in approximately the same stage as No. 9. Many nucleoli show signs of dissolution (Fig. 3).

No. 9. The yolk granules are more rounded than in No. 8 and, especially in the outer cytoplasmic regions, appear somewhat larger. In between these granules there are occasional open spaces, a feature common to oocytes in the later stages of maturation. The nucleus is somewhat more rounded than in No. 8, but nucleoli are not generally present.

Stage G

Mature egg, shortly before oviposition. Animal No. 10.

No. 10. The cytoplasm is like that of No. 9, but the very much shrunken nucleus and the total absence of nucleoli rank these oocytes with certainty as the most mature ones studied.

Evaluation of track counts.—For the testing of differences and correlations, distribution-free tests were used, viz. the median test and the rank correlation test by Kendall [15] respectively, because the parent populations quite clearly did not show a normal distribution. For the estimation of the track density and its variations, *median* and *interquartile range* were indicated; these and the various *P*-values for differences and correlations are assembled in Fig. 4.

Where track counts from two different animals were compared, these were always of necessity derived from two different autoradiographic preparations. It was established by comparing counts for one and the same animal from two or three preparations, that these did not differ significantly in any respect. It does, therefore, not seem likely that any of the observed differences between animals have been caused by uncontrolled variations of preparation of the autoradiographs.

The results are as follows:

(1) Difference between cytoplasm and nucleus or between cytoplasm and nucleolus of the same oocyte was highly significant in all animals.

(2) Difference between nucleus and nucleolus of the same oocyte is significant in stages B, D and F. It seems likely that with a greater number of counts this difference would also have been significant in the other stages.

(3) There is no clear correlation between the incorporation in the nucleus and that in the nucleolus of the same oocyte.

(4) The *P*-values indicating the significance of differences between stages are indicated below the graph (Fig. 4). The *cytoplasm* of stage A has a significantly higher incorporation than that of all later stages, which among each other do not differ essentially. From the small number of tracks seen over the cytoplasm of the older oocytes, some of those which were found very close to the nuclear membrane may have originated from the nucleus.

Incorporation into the *nucleus* at stage D differs significantly from that in all other stages, which among themselves do not differ significantly.

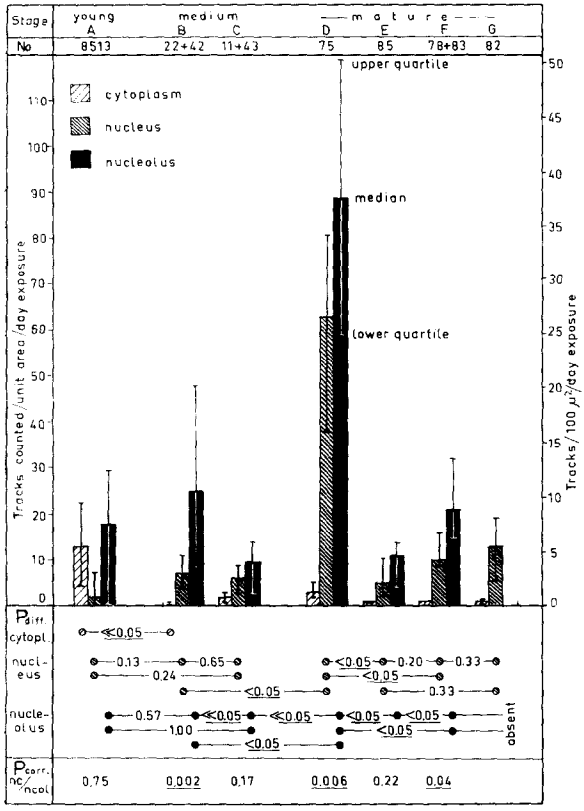

Fig. 4.

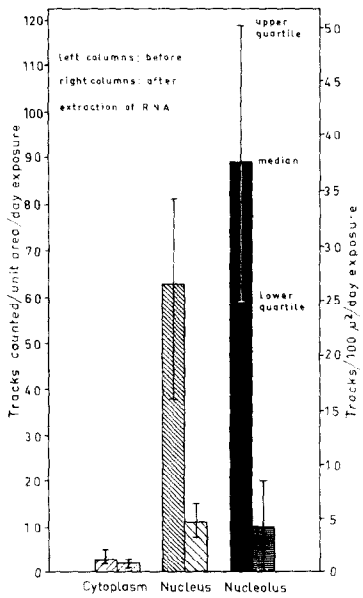

Fig. 5.

Fig. 4.—Track counts indicating incorporation of ^{14}C-adenine into cytoplasm, nucleus and nucleolus of *Asellus* oocytes. Below: *P*-values indicating significance of difference or correlation between track counts.

Fig. 5.—Effect of RNA extraction on track counts of oocytes at stage D.

The picture for the *nucleolus* is more varied. There is a high incorporation in stage B, higher, but not significantly higher than that in the subsequent stage. A second and very high peak is found in stage D, which is significantly higher than all other stages.

Extraction of RNA.—As an example of the result of RNA extraction by means of perchloric acid, counts for animal No. 6 before and after extraction are compared in Fig. 5. Although, by the treatment with 10 per cent perchloric acid at 4°C for 24 hours, the *nucleoli* had lost practically all affinity to pyronine, the track counts are reduced only appr. 90 per cent. It is generally accepted that nucleoli contain very little if any DNA [3], so that the question should be put whether RNA extraction has been complete. On the other hand, Feulgen-positive granules have been observed in the nucleoli of *Asellus* oocytes [16] and small amounts of DNA in nucleoli of other animals are reported [30]. This leaves room for allotting the remaining 10 per cent of tracks to DNA.

The *nuclei* appear to contain a rather large amount of labeled RNA, but the decrease in track count by perchloric acid treatment is significantly smaller than in the nucleoli. Judging from the figures approximately 20 per cent of the label could be ascribed to metabolically active DNA.

In the *cytoplasm* of the oocytes of this and most other stages (except stage A), track counts are so little above background that no accurate estimate can be given of the radioactivity remaining after RNA extraction. It seems likely, however, that the cytoplasm in all cases contains some recently synthesized RNA (see also discussion below).

Sections autoradiographed after treatment with 5 per cent perchloric acid during 1 hr at 60°C to remove both RNA and DNA did not yield additional results. The strong chemographic disturbance, caused by the pyloric caeca and also notable in some other preparations, prohibited the evaluation of these preparations altogether.

DISCUSSION

The survey of the oocyte stages studied (see Table II) has shown that they encompass two main transitional phases of the oocyte, whereas in the interlying, less well-covered period no such transitions occur. A discussion may therefore concentrate on these two transitions.

(1) In the early growth phase (stage A) there is a heavy labelling in the cytoplasm and a slightly less but still prominent labelling in the nucleolus. In the subsequent stage B and C, the cytoplasmic labels is seen to diminish

quickly, whereas the nucleolus shows a significant maximum. In both cases the incorporation can be ascribed almost entirely to RNA metabolism. The question should be put whether cytoplasmic and nucleolar RNA metabolism can be connected with any cytophysiological activity and in how far they are interrelated.

Literature gives many instances (e.g. [3, 11, 12, 13, 28, 32]) where a displacement of RNA from nucleolus to cytoplasm has been demonstrated or made probable. In the *Asellus* oocyte, histological findings [16] strongly point towards a displacement in this direction. The apparent increase of pyronine affinity of the cytoplasm in the earliest stages could support this view and the distribution of β-tracks in the autoradiographs would not contradict it.

On the other hand, the fact that the cytoplasmic maximum precedes that of the nucleolus could point to cytoplasmic RNA synthesis [22, 30] or even to a transport of RNA for cytoplasm to the nucleolus. In so far as this investigation is based on the study of incorporation after only one time interval between isotope injection and death of the animal, these possibilities cannot be ruled out. A further study with other time intervals is now being undertaken.

For the moment, it remains to be judged how far the observations made on the *Asellus* oocyte would fit the pattern drafted for other similar cells, with due consideration of the role of the RNA metabolism in the physiology of the oocyte. The prevailing feature seems to be that the incorporation into the cytoplasm subsides and its pyronine affinity decreases at the end of the second (cytoplasmic growth) period, i.e. when the first yolk vacuoles appear. A cytoplasmic RNA metabolism during the second period may therefore indicate a phase of cytoplasmic activity which, judging from the histological data, might be connected with the initiation of vitellogenesis. Ficq observed a maximum incorporation of ^{14}C-phenylalanine into the cytoplasm and nucleolus in *Asterias* [9] and *Rana fusca* [8] oocytes, in both cases coinciding with the onset of vitellogenesis; the incorporation of adenine, however, showed a steady decline in this period. Roche [23] indicates that the cytoplasm of the *Asellus* oocyte exhibits a very strong alkaline phosphatase reaction only up to this stage, whilst the nucleolus remains very readily stainable by the Gomori method throughout the entire oocyte development. A high alkaline phosphatase activity has been brought into connection with nucleotide synthesis [4], but doubts have been raised as to the validity of such reasoning as far as nucleoli are concerned [30].

Hence, for the cytoplasm there is reason to suggest a connection between

high RNA metabolism and the initiation of vitellogenesis in the second period, but for the nucleolar maximum the connection is less clear. For *Rana fusca* oocytes [8] it has likewise been observed that adenine incorporation in the nucleolus remains high after the maximum in the cytoplasm has passed. A succession of a cytoplasmic by a nucleolar maximum, though less pronounced than in the experiments with ^{14}C-adenine described here, has been observed by Pantelouris [19] for *Triturus* oocytes 6 hours after administration of ^{35}S-methionine. (Such maxima were not observed with shorter or longer time intervals between isotope administration and fixation, but then it is conceivable that they would only appear if this time interval somehow matches the rapidity of the transport of labeled material.)

There are arguments, however, to caution against overemphasizing this succession of maxima in *Asellus*. First, the decline in incorporation in the cytoplasm at the end of the second period could partly be ascribed to a dilution rather than to a decrease of RNA, judging from the appearance of yolk vacuoles at this moment. At approximately the same time, it is seen that the nucleolar vacuoles are depleted and disappear. The intensive labelling of the nucleolar vacuoles with ^{35}S-methionine in *Triturus* oocytes of a similar stage [19] would agree well with the supposition made for the *Asellus* oocyte [16] that at this stage the nucleolus gives off its acidophilic contents, i.e. proteins. Vincent reports that he and Errera observed an absolute increase of RNA in the nucleolus of *Asterias* oocytes even when, owing to its increase in size through vacuolization, the concentration of RNA was declining [30]. In *Asellus*, there is an intense vacuolization of the cytoplasm at the beginning of yolk formation (Fig. 1), whereas nucleolar vacuoles are emptied so that RNA concentration increases. Hence, an essentially constant turnover of nucleolar RNA and/or a constant rate of RNA transport from nucleolus to cytoplasm may, under the conditions prevailing in *Asellus*, be compatible with the appearance of two successive maxima of incorporation as found in the autoradiographs.

The present data do not allow to weigh precisely how far these histological changes can wholly account for the nucleolar maximum succeeding that in the cytoplasm. The assumption that both maxima are connected with the initiation of vitellogenesis seems the simplest explanation covering all observations.

(2) The later stages studied in this investigation cover the end of the third (yolk formation) period and the entire fourth (maturation) period. It is evident that the nucleolus, which after the beginning of the yolk formation

shows a declining incorporation, at the end of this period, i.e. shortly before disappearing during the maturation of the oocyte, again demonstrates a very high activity; this second maximum is even markedly higher than the one observed at the beginning of yolk formation. Apparently much if not all of the ^{14}C-adenine incorporated into the nucleolus is bound to RNA. The presence of a large number of tracks over the nucleus, most of which can also be ascribed to RNA, may be an indication of a transport of this substance through the nucleus to the cytoplasm as well as denote a synthesis of chromosomal RNA. Part of the tracks may, in addition, point to a turnover of DNA.

The number of tracks over the cytoplasm is very low, but, as the measurements cited on page 208 have shown, only less than one-fifth of the cytoplasmic area is taken up by cytoplasm proper. Wherever tracks can be traced back with any certainty in the autoradiographs, they apparently originate from the cytoplasm proper and not from the yolk granules. Hence for stage D, the number of tracks per unit area of cytoplasm proper is of the same order of magnitude as in stage A.

At the end of the maturation period, stages E–G, incorporation in all parts of the oocyte sharply declines and the nucleolus vanishes. In the nucleus, which is then shrunken to an irregular and fairly strongly pyroninophilic mass, the incorporation remains somewhat higher. It could therefore be that RNA synthesized in the nucleolus is passed on to the nucleus where it might be used for the synthesis of DNA. Brachet [3] cites various examples of a synthesis of DNA in the maturing oocyte, where it is distributed through the cytoplasm already before fertilization. As such, the small but undisputable incorporation in the cytoplasm of the final stages in the *Asellus* oocyte might be interpreted. That adenine could have been broken down and ^{14}C be retained in proteins, as was supposed by Ficq for *Rana fusca* [8], does not seem likely after an isotope incorporation period of only 3 hours. Besides formation of DNA out of RNA (also demonstrated for mouse spermatogonia [20]), RNA could be supposed to initiate synthesis of cytoplasmic proteins connected with the development of the fertilized ovum.

Although it is as yet impossible to say which processes underly the incorporation of adenine at this stage, the fact remains that there is a high incorporation just before the oocyte reaches maturity, which makes it reasonable to relate the observed maximum to processes connected with the oocyte's immediate future, viz. fertilization and early development.

Thus, the present study allows us to distinguish two periods of high incorporation of adenine into nucleolar RNA. It appears that each of these

two periods is related to a particular phase in the oocyte's development. The first occurs during the phase of cytoplasmic growth and ends just when the first yolk vacuoles are appearing; the second is found just before the nucleolus disappears and the nucleolar substance is supposedly dispersed through nucleus and/or cytoplasm shortly before the egg leaves the ovary. With reference to the many instances where a direct relation between RNA formation and the synthesis of proteins is suggested, one might see the two maxima of activity in the nucleolus of the *Asellus* oocyte as being related to the formation of enzyme proteins in the cytoplasm; in the first instance such enzymes would then be active in the formation of yolk in the ensuing period, in the second instance other enzymes might become active in the development of the fertilized ovum immediately after its transfer to the breeding pouch. During the actual yolk formation period, the nucleolus seems to be much less active, as far as can be judged from one stage seen, which was located at the beginning of this period and which showed little incorporation in the nucleolus. In contradistinction, the follicle cells appear to be very active throughout the period of yolk formation.

SUMMARY

Asellus aquaticus females were injected with 8-^{14}C-adenine, fixed after 3 hours and sectioned. In coated autoradiographs, the number of β-tracks from ^{14}C were counted over nucleolus, nucleus and cytoplasm of the oocytes at various stages of their development. Incorporation into nucleolar RNA, being high at all stages, was found to be especially prominent in two phases of the oocyte's development. These phases were tentatively connected with, respectively, the onset of yolk formation in the cytoplasm and the full maturation of the oocyte.

The first author is greatly indebted to Mme A. Ficq (Laboratoire de Morphologie animale, Université Libre de Bruxelles) for instructions on the coating method of autoradiography. Both authors wish to express their thanks to Prof. M. T. Jansen for his stimulating interest and to Mr. J. J. Bezem for his expert guidance in the computation of the experimental data. The skilful assistance of Mr. H. Dersjant is gratefully acknowledged.

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