ON THE ORIGIN AND PERSISTENCE OF A CYTOPLASMIC STATE INDUCING NUCLEAR DNA SYNTHESIS IN FROGS' EGGS*

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Nucleocytoplasmic interactions are believed to be of great importance in early animal development, since the response of genetically identical nuclei to different regions of egg cytoplasm provides the most satisfactory explanation for the initial appearance of cell differences.[1-3] However, very little is known at present about the mechanism of nucleocytoplasmic interactions. With this in mind, we have selected an apparently simple example of this kind of interaction for detailed study in the frog *Xenopus laevis*. This is the induction of DNA synthesis by egg cytoplasm, a phenomenon which has been observed in male and female pronuclei shortly after fertilization.[4-20] The analysis of this example of a nucleocytoplasmic interaction is much facilitated by the finding that large numbers of nuclei isolated from frog brain and other adult cell types are rapidly induced to synthesize DNA after injection into unfertilized eggs,[5] though very few nuclei from these tissues normally synthesize DNA. In all experiments to be reported here, nuclei from adult frog brain have been used. Although these nuclei do not support normal development after their injection into unfertilized eggs, they respond to egg cytoplasm by DNA synthesis as do egg and sperm pronuclei or single transplanted embryonic nuclei, which do support normal development.[5] For this reason the induction of DNA synthesis in adult brain nuclei can be justifiably used to study one aspect of normal nucleocytoplasmic interactions.

The experiments reported here contribute to our understanding of this nucleocytoplasmic interaction in three principal ways. First, they show that the state of egg cytoplasm which induces DNA synthesis is totally absent from oöcytes, the cells which mature into eggs. Second, it has been found that this cytoplasmic state arises as an effect of pituitary hormone on mature oöcytes. Last, the experiments demonstrate the persistence of the effective cytoplasmic state which seems to be sufficiently stable to permit eventual identification of its molecular basis.

Materials and Methods.—Preparation and incubation of oöcytes: A suspension of nuclei and label was inserted into oöcytes of *Xenopus laevis laevis* by microinjection (50–100 mμl for full-sized oöcytes and 10–15 mμl for growing oöcytes of half the full size diameter). Injected oöcytes, still surrounded by follicle cells, were incubated at 21°C in modified Barth's medium[5] (i.e., 0.176 *M* NaCl, 2.0 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mg per liter of both streptomycin sulphate and benzylpenicillin sodium salt, and 1.5 mM Tris-HCl to bring the pH of the whole solution to 7.6). Oöcytes incubated under these conditions retained the same external appearance as they had immediately after removal from the donor female for a period varying from 2 to 3 days.

Isolated brain nuclei: These were used as a crude preparation obtained in the way described previously.[5] In any 2-hr labeling period, less than 1% of adult frog brain nuclei synthesize DNA *in vivo*, or as isolated nuclei *in vitro*.[5]

Labeling methods: H^3-thymidine (H^3-TdR) was used as a specific label for DNA synthesis.[5] It was obtained at 22.1 c/mM, labeled primarily in the 6C position, from the Radiochemical Centre, Amersham, England, and was made up at 2 mc/ml in modified Barth's solution. In most experiments it was mixed with brain nuclei (1:1 ratio by volume) just before use.

Autoradiography: Oöcytes or eggs, fixed in Perenyi's fixative and sectioned at 6–7 μ, were

stained and mounted for identification and examination of nuclei. The sections were then taken down to water, treated with cold 5% trichloroacetic acid (TCA) for 20 min and extensively washed out under running water before being dipped in K2 emulsion (Ilford, Essex, England). Exposures were for 3 weeks unless otherwise stated. The great majority of nuclei scored as labeled were densely covered by grains (e.g., Fig. 1*N*, *P*) and all were covered by several times the background number of grains. Nuclei described as unlabeled had no measurably greater number of grains over them than an equivalent area of background (e.g., Fig. 1*J*–*L*).

Hormone: For injection into frogs as well as for addition to oöcyte incubation media, LH obtained as "pregnyl" from Organon Laboratories, Surrey, England, was used to induce ovulation.

Numbers of frogs and eggs used: Each of the main conclusions drawn in this paper is based on experiments carried out on at least 50 oöcytes or eggs obtained from at least two different females.

Normal events accompanying the maturation of oöcytes into eggs: In *Xenopus* the growth of an oöcyte lasts many months or even years; during this time it is attached to the ovary by an enveloping layer of follicle cells and possesses a relatively enormous nucleus called the germinal vesicle (Fig. 1*G*), which contains multiple nucleoli (Fig. 1*E*) and chromosomes in the diplotene stage of meiotic prophase.[7] When oöcytes have reached their full size, they are able to respond to a pituitary hormone, such as LH, by undergoing a series of events called maturation. These include the rupture of the germinal vesicle whose contents mix with the oöcyte cytoplasm, the release of the oöcyte from the ovary (ovulation), and its passage down the uterus; the oöcyte chromosomes complete their first meiosis, releasing a polar body, and proceed as far as the metaphase of the second meiotic division. At this point the matured, ovulated oöcyte is called an egg. All these maturation events can take place in *Xenopus* within 12 hr or less of hormone administration. Eggs, but not oöcytes, respond to penetration (whether provided artificially by pricking with a micropipette, or naturally by sperm during fertilization) by undergoing a process called activation. This involves the completion of the second meiosis, the bursting of the cortical granules, etc. In the experiments described below, living eggs were considered to have undergone activation if they showed irregular fragmentation of animal hemisphere cytoplasm. Eggs, but not oöcytes, always respond in this way to penetration by a micropipette. This sequence of events differs in detail in other anuran species.[8]

Results.—Absence of thymidine incorporation by nuclei injected into oöcytes: When isolated adult brain nuclei and H^3-thymidine are injected into unfertilized eggs, over 90 per cent of them are observed by autoradiography to have incorporated the label into DNA within 90 minutes[5] (Fig. 1*O*, *P*). Brain nuclei prepared in the usual way were injected together with the label into the cytoplasm of full-sized oöcytes which were fixed 90 minutes later. Exposure to autoradiographic film for the same length of time as eggs revealed no grains over the nuclei. The oöcytes which received the injected nuclei retained a normal external appearance *in vitro* for 2–3 days, and the injected nuclei remained indistinguishable from nuclei in fixed brain tissue for several hours after injection into oöcytes. Even when nuclei were allowed to remain in oöcytes for 3 days after injection at the same time as H^3-thymidine, no DNA synthesis could be detected. Similar experiments have been carried out on young oöcytes that are growing very actively. These were obtained from a female which had been induced by pituitary hormone to spawn large numbers of eggs 3 days before. After females have spawned, their young oöcytes are very active in growth and RNA synthesis.[9, 10] Brain nuclei injected together with H^3-thymidine into oöcytes of half the full-size diameter (in the active lampbrush phase) showed no DNA synthesis, even if left in the oöcytes for 2–3 days and if exposed to autoradiographic film for 2–3 months (Fig. 1*A*, *E*, *J*).

It seemed possible that the factor responsible for the induction of DNA synthesis by egg cytoplasm might accumulate in the oöcyte nucleus (germinal vesicle) and be liberated into the cytoplasm when the germinal vesicle ruptures just before

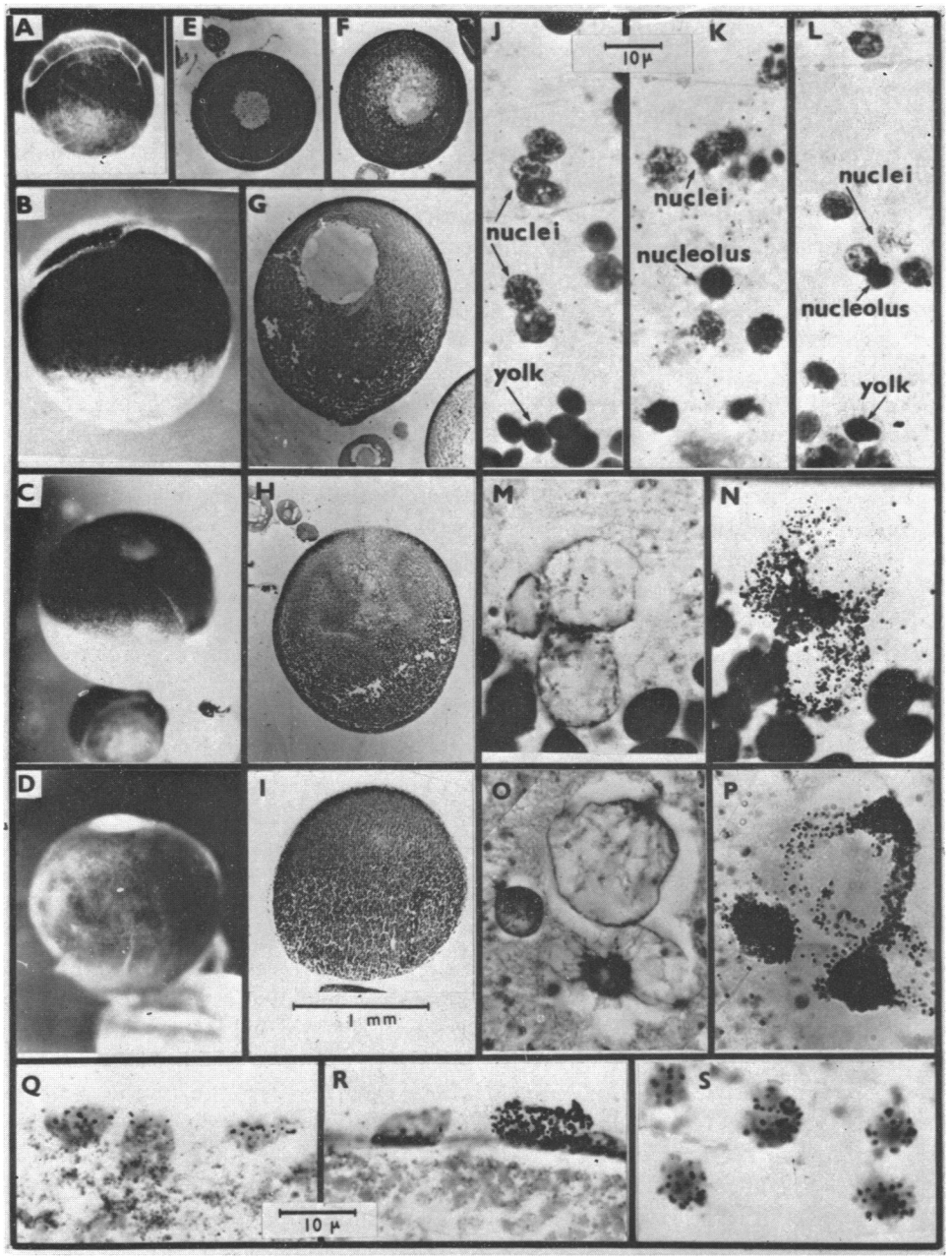

FIG. 1.—(*A–D*) Whole oöcytes or eggs; (*E–I*) vertical sections; (*J–P*) adult brain nuclei 90 min after injection at the same time as H^3-thymidine. (*A, E, F*) Young oöcyte; in (*F*) germinal vesicle sap has been mixed with oöcyte cytoplasm; (*B, G*) full-sized oöcyte; (*C, H*) nonovulated oöcyte with ruptured germinal vesicle; (*D, I*) unfertilized egg. Magnification in (*A*)–(*I*) is the same as in (*I*). (*J, K, L*) Autoradiographs of nuclei in oöcyte cytoplasm (*J*), in an oöcyte germinal vesicle (*K*), and in oöcyte cytoplasm mixed with germinal vesicle contents (*L*); (*M, N*) nuclei in an oöcyte with a ruptured germinal vesicle (as in *C, H*) before and after autoradiography; (*O, P*) nuclei in an unfertilized egg before and after autoradiography. Magnification in (*J–P*) and (*S*) is the same as in (*J*) and (*K*). (*Q, R*) Oöcyte follicle cell nuclei all of which have incorporated H^3-uridine (*Q*), and only some of which have incorporated H^3-thymidine (*R*). (*S*) H^3-uridine incorporation by nucleoli of injected oöcytes which were maintained *in vitro* for 3 hr.

ovulation. This was tested by injecting brain nuclei and H^3-thymidine *inside* the germinal vesicle. In the case of small oöcytes taken from a female which had spawned 3 days earlier, it was found that 10–20 per cent of the injected oöcytes contained apparently normal brain nuclei in the germinal vesicle. Oöcytes of this kind were incubated *in vitro* for 6 hours, but no incorporation of H^3-thymidine was observed in either the resident germinal vesicle or the injected nuclei (Fig. 1*K*). It was not found possible to introduce nuclei into the germinal vesicle of *mature* oöcytes by the same means, since in *Xenopus laevis* the germinal vesicle sap of full-sized oöcytes has a stiff jelly-like consistency, and the nuclei which can be assumed to have been deposited in the germinal vesicle are believed to be squeezed out into the oöcyte cytoplasm soon after withdrawal of the microinjection pipette. For this reason, germinal vesicles were dissected out of full-sized oöcytes, injected with nuclei mixed with H^3-thymidine, and incubated as isolated germinal vesicles. Although in most cases nuclei were observed to be quickly squeezed out through the injection pore, some germinal vesicles retained the injected nuclei and were incubated *in vitro* for $1^1/_2$ hours with H^3-thymidine added to the medium. However, no incorporation of H^3-thymidine was observed in any of the nuclei.

There remained the possibility that the factor promoting DNA synthesis is formed or activated by the interaction of a component inside the germinal vesicle with another in the oöcyte cytoplasm, and would therefore not normally appear until germinal vesicle breakdown. This possibility was excluded by reference to certain injected young oöcytes in which a substantial leakage of germinal vesicle contents had taken place. Injected nuclei present in the region where the germinal vesicle contents had mixed with the oöcyte cytoplasm showed no H^3-thymidine incorporation (Fig. 1*F*, *L*).

Appearance, during oöcyte maturation, of the cytoplasmic state inducing thymidine incorporation: It has been possible to determine the extent to which the different processes constituting oöcyte maturation (see *Methods*) contribute to the appearance of the cytoplasmic state which induces DNA synthesis. The germinal vesicle ruptures and disperses its contents before the egg is released from the ovary and before the meiotic divisions of the nucleus have commenced. Eggs in which the germinal vesicle has burst but which have not been ovulated can be recognized by their external appearance (Fig. 1*C*), and brain nuclei injected into such eggs show extensive incorporation of H^3-thymidine within $1^1/_2$ hours. Evidently, the factor promoting DNA synthesis appears at the same time as rupture of the germinal vesicle. That this temporal association is not fortuitous is indicated by finding injected nuclei in occasional eggs which had been ovulated, passed through the oviduct, and laid as usual, but in which the germinal vesicle had failed to break down; nuclei injected into such eggs always fail to incorporate the label.

Reasons for the absence of thymidine incorporation by nuclei injected into oöcytes: The results reported so far indicate that at least some of the conditions which promote DNA synthesis in eggs are absent from oöcytes. However, the inability to demonstrate DNA synthesis by nuclei injected into oöcytes could be attributed to any of the following causes: (1) the injected H^3-thymidine might not be used for any DNA synthesis which is in fact taking place, (2) the recipient oöcytes might not remain synthetically active after removal from the ovary and during incubation *in vitro*, or (3) the isolated brain nuclei might be damaged or permanently inactivated

by contact with oöcyte cytoplasm. These possibilities have been excluded by the following experiments.

Using Dowex-1-formate chromatography, Woodland (unpublished) has found that about 70 per cent of the H^3-thymidine injected into oöcytes is converted to thymidine triphosphate (TTP) when the oöcytes are maintained *in vitro* for 3 hours. This shows that the enzymes required for thymidine phosphorylation (thymidine kinase and thymidine monophosphate (TMP) + thymidine diphosphate (TDP) kinase) are present and active in oöcytes. Evidence that the labeled TTP is in fact available for incorporation into DNA is conveniently provided by the follicle cells which surround each oöcyte. About 10–20 per cent of these follicle cell nuclei are labeled intensely with H^3-thymidine after incubation of oöcytes *in vitro* for $1^1/_2$ hours (Fig. 1*R*). Since the medium in which the oöcytes are incubated contains no added H^3-thymidine and only a very low concentration of label by leakage from injected oöcytes, the follicle cells evidently obtain most of their label by diffusion from the oöcyte cytoplasm. This shows that the injected H^3-thymidine is not sequestered by or adsorbed onto oöcyte components, but is freely available for incorporation into DNA. The isolated injected oöcytes are known to stay alive and synthetically active *in vitro* from the fact that all the germinal vesicle nucleoli of growing oöcytes incorporate H^3-uridine into RNA (Fig. 1*S*), just as they do *in vivo*.

That the inability to demonstrate DNA synthesis by nuclei in oöcytes is not due to a dilution of the injected H^3-thymidine in a large DNA precursor pool or to the inviability of injected nuclei has been demonstrated by an experiment of the following design. Brain nuclei mixed with H^3-thymidine were injected into full-sized oöcytes of a female which was about to commence ovulation following hormone administration. The oöcytes selected for injection had intact germinal vesicles as was ensured by the absence of the white animal pole region (seen in Fig. 1*C*) and by the fact that intact germinal vesicles could be dissected out of oöcytes with an external appearance similar to those in Figure 1*B*. The injected oöcytes were then incubated in medium containing 20 IU of LH hormone per milliliter. Many of the incubated oöcytes matured *in vitro* as has been found before for other species of Amphibia,[11] and their germinal vesicles ruptured. When this was observed by external appearance (Fig. 1*C*) to have taken place, the eggs were incubated for a further $1^1/_2$ hours and then fixed. Many of the brain nuclei in these *in vitro* matured eggs did not synthesize DNA but contained highly condensed chromosomes and entered an abortive meiosis or mitosis to be described elsewhere. However, an appreciable number of nuclei in most eggs were vesicular in appearance and had swollen to some extent (Fig. 1*M*). These incorporated H^3-thymidine into DNA (Fig. 1*N*). Some of the injected oöcytes which were treated identically to those above failed to mature *in vitro*, and retained an intact germinal vesicle; in these, no incorporation was observed in any of the injected nuclei. Apart from confirming that the appearance of the condition inducing DNA synthesis is precisely associated in time with germinal vesicle breakdown, these experiments demonstrate that the absence of DNA synthesis in oöcytes cannot be explained either as damage to the injected nuclei or as a dilution of the injected H^3-thymidine in a large precursor pool. Thus, brain nuclei injected into oöcytes remain viable for at least several hours, and are capable of synthesizing DNA as soon as the germinal

vesicle ruptures. The labeled thymidine will be diluted in the oöcyte pools of TdR, TMP, TDP, and TTP soon after injection, and yet the precursor pool is still sufficiently labeled to clearly reveal DNA synthesis when the same oöcytes have undergone maturation and germinal vesicle breakdown. In conclusion, the inability to demonstrate H^3-thymidine incorporation by nuclei in oöcytes evidently reflects a true lack of conditions necessary for DNA synthesis.

Persistence of the cytoplasmic state responsible for the induction of DNA synthesis: In order to identify the cytoplasmic component which induces DNA synthesis, it is important to know whether it has a transient existence and disappears soon after it arises, or whether it remains in an effective state for a considerable time after fertilization. Two kinds of experiments have been used to answer this question.

In the first experiment, brain nuclei were injected into eggs which then received a 20-minute pulse of H^3-TdR by a second injection at various times after this (Fig. 2*E–I*). The conclusion from these experiments is that egg cytoplasm is able to

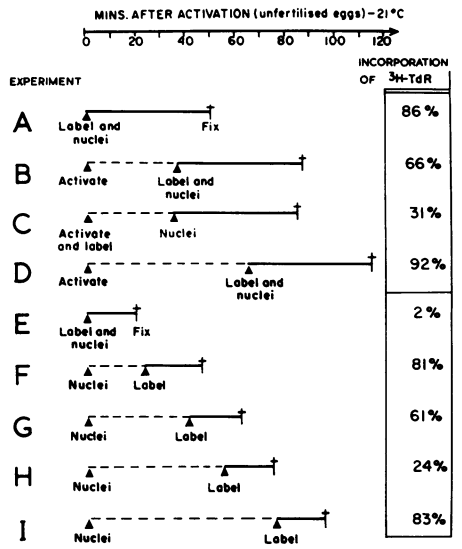

FIG. 2.—Design of experiments to test persistence of DNA synthesis-inducing factor. H^3-TdR, H^3-thymidine. The low percentage of labeled nuclei in experiment *H* (24%) may be due to the fact that many nuclei were not properly mixed with cytoplasm in these eggs. Two hundred nuclei were scored for each per cent value.

support DNA synthesis from 20 to at least 100 minutes after nuclear injection or egg activation. Furthermore, many of the *same* nuclei synthesize DNA during most or all of this period (compare Fig. 2*F*, *G*, *I*). The absence of DNA synthesis for the first 20 minutes after fertilization is characteristic of male and female pronuclei.[4] It seems that these, as well as adult somatic cell nuclei, require a certain time to respond to their new cytoplasmic environment. It is during this time that nuclei undergo a pronounced swelling[5] and that their DNA is thought to undergo a change in state or in degree of dispersion.[12]

In the second type of experiment, egg cytoplasm was shown to have a persistent capacity not only to support but also to initiate DNA synthesis. This was demonstrated by activating unfertilized eggs (with a prick), and then injecting nuclei at various times after this. As shown in Figure 2*A–D*, egg cytoplasm retains its ability to initiate DNA synthesis for at least 65 minutes after egg activation, whether label was introduced at activation or with the nuclei.

Discussion.—A cytoplasmic factor responsible for the induction of DNA synthesis can now be added to the known examples of substances which are formed during oögenesis or egg maturation but which do not normally become effective until after fertilization. In *Xenopus laevis*, ribosomes synthesized during oögenesis are sufficient for the needs of cell differentiation up to the swimming tadpole stage.[13] In the axolotl, Briggs and Cassens[14] have provided a very clear demonstration that normal postgastrular development requires a gene product synthesized during oögenesis. However, more comparable with the DNA synthesis factor described here for *Xenopus*, at least in its time of appearance and activity, is the factor required for cleavage and aster formation in many animal eggs. It has been known for a long time that the ability of various invertebrate eggs to be fertilized arises only after rupture of the germinal vesicle.[15] In *Bufo*, Dettlaff *et al.*[11] have demonstrated by a variety of experiments that a factor which is necessary for egg activation and cleavage, and which might include a DNA synthesis inducer, appears at the time of germinal vesicle breakdown and was not present before in the germinal vesicle or oöcyte cytoplasm. The cleavage and activation response is realized in *Bufo* only if the eggs are activated by pricking *after* the release of the germinal vesicle contents.[11] This is not true of the DNA synthesis inducer in *Xenopus*, which as shown above is operative in eggs which matured *in vitro* and which are not pricked after germinal vesicle breakdown.

The induction of DNA synthesis by egg cytoplasm may be regarded as a simple form of a type of nucleocytoplasmic interaction that is of great importance in early development. It is a reaction of which we know the product (DNA) and which may be controlled by the availability of the essential reacting components. The following sequence of events seems to account most simply for the control of the reaction. The required component appears as an effect of pituitary hormone on mature oöcytes, and this is likely to involve both protein synthesis and DNA-dependent RNA synthesis, since Dettlaff[16] has shown that germinal vesicle breakdown in *Bufo* is both puromycin- and actinomycin D-sensitive. Smith *et al.*[17] have observed a great increase in the rate of protein synthesis at this time in *Rana*. The induction of DNA synthesis cannot be explained solely as a direct effect of the hormone on the injected nuclei, because it is not induced in an incubation of isolated nuclei to which hormone has been added, nor by the microinjection of hormone as well as nuclei and H^3-thymidine into full-sized oöcytes with an intact germinal vesicle (Gurdon, unpublished observations). It is of special interest that in *Xenopus* the DNA synthesis-inducing factor can first be demonstrated at just the time when the oöcyte chromosomes enter an unresponsive, condensed state in which they remain during meiosis until fertilization stimulates them to proceed beyond the second meiotic metaphase. Since they have reached at least the 4C condition by meiotic prophase,[18, 19] they do not need to synthesize DNA during oöcyte maturation, and condensed mitotic or meiotic chromosomes have never been reported to do so. Thus, it seems that the regulation of this example of a nucleocytoplasmic interaction can at present be adequately accounted for by precise control of the time at which responsive chromosomes are exposed to the cytoplasmic factor. The situation may well turn out to be more complicated than suggested here, and we have in any case to explain what causes chromosome condensation and the continuation of meiosis at the time of germinal vesicle breakdown.

Perhaps the most interesting aspect of this reaction, not yet understood, is the identity of the DNA synthesis-inducing factor. Little is known about this at present except that it is not species-specific,[5] and that it appears not to include the enzymes necessary for thymidine phosphorylation (see above).

Summary.—Adult frog brain nuclei have been injected into oöcytes at various stages of growth and maturation in order to determine the stage at which egg cytoplasm acquires its capacity to induce DNA synthesis in gamete and other nuclei. DNA synthesis was recognized autoradiographically by incorporation of H^3-thymidine. The factor which induces DNA synthesis is absent from the nucleus and cytoplasm of growing oöcytes and from a mixture of nucleus and cytoplasm. The factor appears a few hours after the administration of pituitary hormone, that is, just after the rupture of the oöcyte nucleus. It persists in the egg cytoplasm for at least an hour after fertilization or activation, but is effective in the absence of fertilization.

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