

On the 'clock' mechanism determining the time of tissue-specific enzyme development during ascidian embryogenesis

II. Evidence for association of the clock with the cycle of DNA replication

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SUMMARY

Acetylcholinesterase (AChE) is a tissue-specific enzyme of the muscle cells of ascidian embryos and its synthesis begins at the neurula stage. Embryos which had been permanently cleavage-arrested with cytochalasin B could develop AChE activity. The time of first AChE occurrence in embryos which had been arrested in the 32-cell stage with cytochalasin was about the same as in normal embryos. The nucleus in the cell of cytochalasin-arrested embryos divided in good synchrony with that of normal embryos. Embryos which had been continuously arrested with colchicine could also produce AChE activity at nearly the same time as did normal embryos. In the cell of colchicine-arrested embryos normal nuclear divisions did not occur, but the cell showed repeated cycles of nuclear envelope breakdown and nuclear envelope reformation in almost parallel with cell cycles of normal embryos. The cell of colchicine-arrested embryos incorporated [³H]thymidine. Aphidicolin, a specific inhibitor of DNA synthesis, prevented cleavages of ascidian eggs. Embryos which had been permanently arrested with aphidicolin in the cleavage stages up to the 64-cell stage did not develop AChE activity, while embryos which had been treated with it from the 76-cell stage onwards were found to be able to differentiate AChE activity. Based on these findings it was proposed that DNA replication is prerequisite for development of the histospecific protein and that the cycle of DNA replication is closely associated with the clock mechanism which is determining the time of initiation of the enzyme development.

INTRODUCTION

During ascidian embryogenesis a tissue-specific enzyme, muscle acetylcholinesterase (AChE), is first detected histochemically in the presumptive muscle cells of the neurula (Durante, 1956; Whittaker, 1973; Meedel & Whittaker, 1979; Satoh, 1979). Since there are distinct and separate actinomycin D-

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and puromycin-sensitivity periods for the occurrence of AChE activity, the histospecific protein is thought to be synthesized at that stage with activation of appropriate genes (Whittaker, 1973, 1977; Meedel & Whittaker, 1979; Satoh, 1979). In the preceding study (Satoh, 1979), in order to investigate a 'clock' or counting mechanism that is determining the time when AChE first appears, Whittaker's experiment (1973) has been repeated. Since embryos which had been permanently cleavage-arrested with cytochalasin B were able to differentiate AChE, the time is not apparently regulated by the events of cytokinesis. Embryos which had been arrested with colchicine or with colcemid could also develop AChE activity. Judging from this result the clock does not seem to be controlled by the mitotic cycle of the nucleus.

The present investigation was undertaken to explore the possibility that the cycle of DNA replication is related to the regulation of the time. At first, changes in the morphology of the nucleus related to the cleavage cycles were reviewed in embryos which had been arrested with cytochalasin or with colchicine. Secondly, whether DNA synthesis takes place in the cells of colchicine-arrested embryos or not was determined. Thirdly, effects of inhibitors of DNA synthesis on AChE development were examined. All the results obtained demonstrated that DNA replication is prerequisite for the histospecific enzyme development.

MATERIALS AND METHODS

Animals

Eggs of two ascidian species, *Ciona intestinalis* and *Halocynthia roretzi*, were used in this study. Artificially fertilized eggs of *C. intestinalis* were raised in filtered sea water at 18 ± 0.2 °C using a Yamato Lo-Temp water bath, and those of *H. roretzi* at 15 ± 0.2 °C. Only batches in which cleavage occurred in more than 95% of the eggs were used. Development of eggs from different animals fertilized at the same time is essentially synchronous in each species. The timing of the developmental stages of each species has been shown in the previous reports (Whittaker, 1973; Satoh, 1979).

Enzyme histochemistry

Embryos for acetylcholinesterase (AChE) reactions were fixed for several min in 5% formalin sea water and treated for 2–4 h at 37 °C by the direct-colouring thiocholine method (Karnovsky & Roots, 1964). Reacting specimens were dehydrated in ethanol, cleared in xylene, and embedded in balsam for permanent whole mounts.

Cleavage inhibition

Cytochalasin B (Aldrich Chem. Co.) at 2 µg/ml and colchicine (Sigma Chem. Co.) at 200 µg/ml were used as cleavage inhibitors.

Paraffin section

To examine changes in the morphology of the nucleus related to cleavage cycles, cleavage-arrested embryos as well as normal embryos were fixed in Bouin's solution. They were serially sectioned in paraffin at 8 μm , and stained with haematoxylin and eosin.

DNA synthesis in the cells of colchicine-arrested embryos

[³H]Thymidine incorporation. *Ciona* embryos at the 64-cell stage were incubated in 5 ml of sea water containing both 200 $\mu\text{g/ml}$ colchicine and [³H]thymidine (10 μCi , 46 Ci/mmol; The Radiochemical Centre Ltd). Incorporation was stopped by pouring embryos into ice-cold sea water 1, 2 and 3 h after commencement of incubation, respectively. The embryos were collected by centrifugation and washed four times with ice-cold sea water acidified to pH 5.0 with 0.1 N-HCl. This washing procedure removes the outer follicle cells, which are the principal source of possible bacterial contamination (Meedel & Whittaker, 1978). After washing the embryos were fixed and extracted with 10% trichloroacetic acid (TCA), and then acid-insoluble radioactivity was counted in a liquid scintillation counter.

Autoradiography. *Ciona* embryos were treated for 2 h with colchicine (200 $\mu\text{g/ml}$) in the presence of [³H]thymidine. Treated embryos were fixed in Bouin's solution. They were dehydrated in ethanol, cleared in xylene and embedded in paraffin. Slides of mounted 8 μm sections were coated with Sakura NR-M2 dipping emulsion. Exposure was for 3–5 weeks at 5 °C. Developed autoradiographs were stained with hematoxylin and eosin.

Inhibition of DNA synthesis

Cytosine arabinoside (Sigma Chem. Co.), mitomycin C (Kyowa Hakko Chem. Co.), hydroxyurea (Wako Chem. Co.) and aphidicolin were used as inhibitors of DNA synthesis. Recently it has been verified that aphidicolin, a tetracyclic diterpene-tetraol produced by several fungi, blocks DNA synthesis in sea urchin embryos by interfering with the activity of DNA polymerase- α (Ikegami *et al.* 1978). Effects of aphidicolin on DNA, RNA and protein synthesis in ascidian embryos were examined by incorporation of labelled components.

[³H]Thymidine incorporation. About 3000 *Ciona* embryos were treated for 30 min with aphidicolin and subsequently reared during h 5–7 of development in 5 ml of sea water containing both 10 $\mu\text{g/ml}$ aphidicolin and [*methyl-³H*]thymidine (25 μCi , 46 Ci/mmol). After incubation the embryos were washed four times with ice-cold acidified sea water (pH 5.0). The embryos were then fixed and extracted with 10% TCA, and the acid-insoluble radioactivity was counted in a liquid scintillation counter.

[¹⁴C]Uridine incorporation. About 3,000 *Ciona* embryos were treated for 30 min with aphidicolin and subsequently reared during h 6–8 of development in 5 ml of sea water containing both 10 µg/ml aphidicolin and [U-¹⁴C]uridine (1 µCi, 490 mCi/mmol; The Radiochemical Centre Ltd). Incorporation was stopped by pouring embryos into ice-cold acidified sea water (pH 5.0), and the embryos were washed four times with ice-cold acidified sea water. The embryos were then fixed and extracted with 10% TCA, and the acid-insoluble radioactivity was counted in a liquid scintillation counter.

L-[³H]Leucine incorporation. About 3000 *Ciona* embryos were incubated during h 7–9 of development in 5 ml of sea water containing both 10 µg/ml aphidicolin and L-[4,5-³H]leucine (20 µCi, 170 Ci/mmol; The Radiochemical Centre Ltd). After incubation the embryos were washed four times with ice-cold acidified sea water (pH 5.0). The incorporation of L-[³H]leucine into proteins was determined by the filter paper method of Mans & Novelli (1961). After the final washing the embryos were suspended in 1 ml of cold distilled water and homogenized in a Teflon homogenizer. One tenth ml aliquots of the homogenates were pipetted onto each of two discs of Whatman's MM filter paper. The discs were dried under a hair dryer. One disc was counted in a liquid scintillation counter without further treatment in order to determine the total amount of precursor taken up by embryos (the results were not shown in this report). The second disc was extracted two times, for 20 min each, in 5% TCA at 80 °C and then extracted two times with 10% TCA in the cold to remove low-molecular-weight precursors and nucleic acid. The disc was then extracted once for 1 h in a solution of 95% ethanol and ether (1:1 by volume) at 37 °C to remove lipids, and then washed once with the alcohol and ether solution and then twice with ether. The disc was then dried completely under a hair dryer and counted in a liquid scintillation counter.

RESULTS

Cyclical changes in the morphology of the nucleus in the cleavage-arrested embryos

As revealed in the preceding studies, the time of first AChE occurrence in embryos that had been permanently cleavage-arrested in the 32-cell stage with cytochalasin B was about the same as in normal embryos (Whittaker, 1973; Satoh, 1979). *Halocynthia* embryos which had been cleavage-arrested in the 4-cell stage with cytochalasin as well as normal embryos were fixed at 10 min intervals for 2.5 h (i.e. about three cell cycles) and cyclical changes in the morphology of the nucleus were compared in paraffin-sectioned specimens between the cytochalasin-arrested embryos and normal embryos. The results are shown in Fig. 1 (I and II). *Halocynthia* eggs divide every 45–50 min up to the 32-cell stage at 15 °C. About 10 min prior to cytokinesis of blastomeres of normal embryos, the nuclear envelope disappeared and spindle formed (Fig. 1,

Ib). After division the nuclear envelope reappeared in each daughter cell (Fig. 1, Ic). These changes repeated cyclically in the succeeding cleavage cycles (Fig. 1, d-g). In the cell of cytochalasin-arrested embryos, cyclical breakdown and reformation of the nuclear envelope and spindle formation took place similarly to normal embryos, except that cytokinesis did not occur, resulting in a multinucleated cell (Fig. 1, IIa-g). The time schedule of these cyclical changes in cytochalasin-arrested embryos was almost the same as in normal embryos.

Whittaker (1973) reported that *Ciona* embryos which had been arrested with colchicine or with colcemid in the 2-cell stage and later cleavage stages could develop AChE activity. The present study confirmed this result. The time of first AChE occurrence in *Ciona* embryos that had been arrested in the 32-cell stage with colchicine was nearly the same as in normal embryos. Changes in the morphology of the nucleus in colchicine-arrested *Ciona* embryos were examined in the materials fixed at 10 min intervals for 1.5 h (three cell cycles between the 8-cell and 32-cell stage) and compared to those of normal embryos. The results are also shown in Fig. 1 (III and IV). *Ciona* eggs divide about every 40 min during these cleavage cycles at 18 °C (Fig. 1, III). In colchicine-arrested embryos the cell did not form a spindle or divide (Fig. 1, IV). The so-called nuclear divisions were not observed in the cells of colchicine-arrested embryos. The nuclear envelope, however, cyclically broke down (Fig. 1, IVa, c, d, f) and later reformed (Fig. 1, IVb, e, g). In this case several small nuclei or karyomeres reappeared instead of a single large nucleus (Fig. 1, IVb, e, g). These events, presumably parallel those in normal nuclear reconstitution, took place nearly in synchrony with those in normal embryos, although 10–20 min delay of reappearance of karyomeres was observed in some of colchicine-arrested embryos.

In the case of *Halocynthia* embryos the early gastrula which had been prevented from undergoing further cell division by being left in colchicine or in colcemid could develop AChE activity, but no histochemically detectable AChE activity was found in the cleavage-stage embryos that had been arrested with either drug. Such cyclical disappearance–reappearance of the nuclear envelope as seen in colchicine-arrested *Ciona* embryos was not observed in *Halocynthia* embryos which had been arrested in the 8-cell stage with colchicine.

DNA synthesis in the cells of colchicine-arrested embryos

To determine whether or not DNA synthesis takes place in the cells of colchicine-treated embryos, incorporation of [³H]thymidine was compared between normal and colchicine-arrested embryos. As shown in Table 1, the cells of colchicine-treated embryos incorporated during 1 h incubation almost the same amount of labelled thymidine as did control embryos. In addition, the radioactivity count in colchicine-arrested embryos increased progressively with incubation time (Table 1). There were only slight differences between control and experimental embryos in thymidine incorporation (Table 1). Since

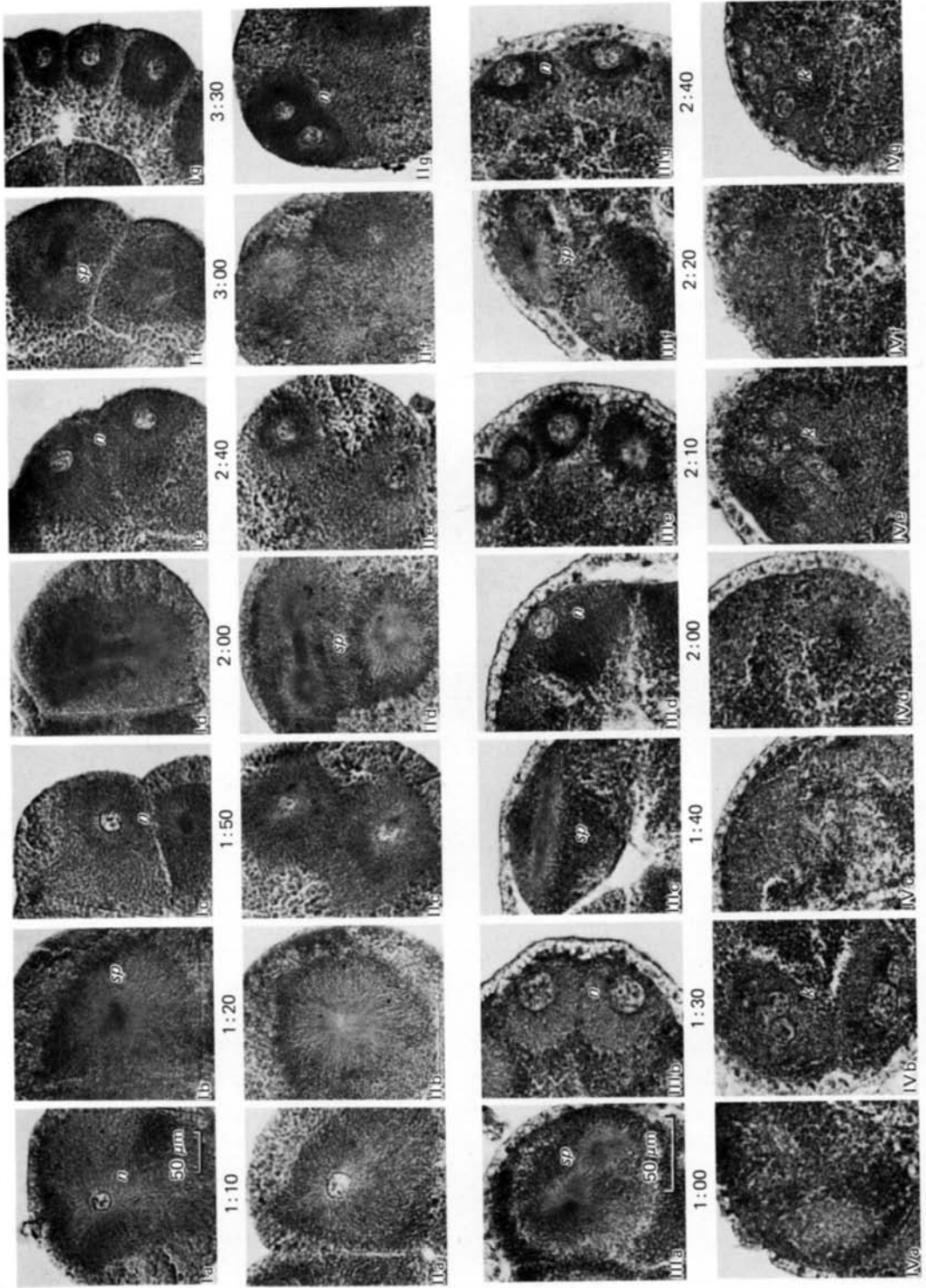


Table 1. [^3H]Thymidine incorporation in the cells of colchicine-arrested embryos of *Ciona intestinalis*

Time of incubation (h)	Experiment 1 ($\times 10^{-1}$ c.p.m./embryo)		Experiment 2 ($\times 10^{-1}$ c.p.m./embryo)	
	Control	Colchicine	Control	Colchicine
1	9.75 (100%)	10.58 (101%)	10.78 (100%)	8.56 (79%)
2	24.61 (252%)	29.97 (307%)	26.23 (243%)	18.48 (171%)
3	32.78 (336%)	46.14 (413%)	30.50 (285%)	28.70 (266%)

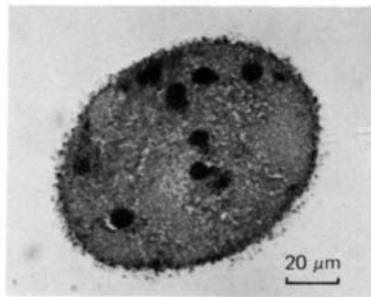


Fig. 2. Autoradiograph of *Ciona* embryo which has been cleavage-arrested with colchicine in the presence of [^3H]thymidine, showing that labelled thymidine incorporation takes place almost exclusively in the nuclei or karyomeres of embryonic cells and not in test cells.

the ascidian embryo is surrounded by test cells within the chorion, it was necessary to determine whether the site of [^3H]thymidine incorporation was in embryonic cells or in accessory cells. This question was resolved with autoradiography. Fig. 2 shows that thymidine incorporation took place almost exclusively in embryonic cells and not in test cells.

These results indicate that colchicine prevents both cytokinesis and karyokinesis in cells applied to it, but not cyclical disappearance–reappearance of the nuclear envelope and DNA synthesis as well.

Fig. 1. Cyclical changes in the morphology of the nucleus in cleavage-arrested embryos. (I) *Halocynthia* normal embryos and (II) *Halocynthia* embryos which have been permanently cleavage-arrested with cytochalasin B in the 4-cell stage. *n*, Nucleus; *sp*, spindle. Numerals between the two series of photographs indicate time after the first cleavage. Cytochalasin treatment begins 50 min after the first cleavage. The nucleus of cells treated with cytochalasin continues dividing in good synchrony with that of normal embryos. (III) *Ciona* normal embryos and (IV) *Ciona* embryos which have been arrested with colchicine in the 4-cell stage. *k*, Karyomeres. Colchicine treatment begins 40 min after the first cleavage. In the cell of colchicine-treated embryos the so-called nuclear division does not occur, but the cell shows repeated cycles of nuclear envelope breakdown and nuclear envelope reformation.

Table 2. *Effects of aphidicolin (10 µg/ml) on DNA, RNA and protein synthesis in the ascidian embryos (Ciona intestinalis)*

Experiment	³ H]Thymidine incorporation (× 10 ⁻¹ c.p.m./embryo)		¹⁴ C]Uridine incorporation (× 10 ⁻² c.p.m./embryo)		L- ³ H]Leucine incorporation (c.p.m./embryo)	
	Control	Aphidicolin	Control	Aphidicolin	Control	Aphidicolin
A	65.66	1.49 (2%)	20.81	15.51 (75%)	13.88	15.64 (113%)
B	73.48	1.58 (2%)	21.78	14.40 (66%)	13.65	15.58 (114%)
C	51.21	0.91 (2%)	25.61	19.38 (76%)	13.78	13.69 (99%)

Inhibition of DNA synthesis and AChE development

In order to determine whether or not DNA replication activity is indispensable and/or prerequisite for the occurrence of AChE activity, effects of several inhibitors of DNA synthesis on cleavage of ascidian eggs and AChE development were examined. Cytosine arabinoside, mitomycin C, and hydroxyurea, however, did not have any effect on development of *Ciona* embryos at any concentration. But, aphidicolin at 2–10 µg/ml prevented divisions of both *Ciona* and *Halocynthia* eggs. Aphidicolin allowed one more cleavage in eggs kept in aphidicolin and stopped divisions thereafter, no matter how soon after a cleavage it was applied. Whether DNA synthesis is inhibited by the treatment with aphidicolin was examined by [³H]thymidine incorporation. Effects of aphidicolin on RNA and protein synthesis were also examined. The results are summarized in Table 2. When *Ciona* embryos were treated for 30 min with aphidicolin (10 µg/ml) and subsequently reared for 2 h in sea water containing both aphidicolin and [³H]thymidine, the incorporation of labelled thymidine into DNA of the embryos was inhibited by 98% (Table 1). If aphidicolin treatment begins in the presence of [³H]thymidine, aphidicolin inhibited more than 90% of labelled thymidine incorporation in the ascidian embryos. Aphidicolin at 10 µg/ml caused about 25% inhibition of labelled uridine incorporation into the acid-insoluble material, but the rate of protein synthesis was the same whether or not embryos were exposed to the compound (Table 1).

In *Ciona* embryos aphidicolin prevented the occurrence of AChE activity in the embryos treated continuously with it from the 64-cell stage onwards. Some embryos treated continuously with aphidicolin from the 76-cell stage onwards eventually developed distinct traces of enzyme activity, and almost all the embryos treated from the early gastrula stage produced a large amount of AChE activity. A similar result was obtained in *Halocynthia* embryos. The embryos that had been permanently cleavage-arrested with aphidicolin in the

cleavage stages up to the 64-cell stage could not develop AChE activity. Embryos which had been arrested with the drug in the early gastrula stage were able to differentiate distinct AChE activity.

Since embryos which had been continuously cleavage-arrested with aphidicolin for 30 min or more could not recover cell-division activity, experiments in which several cell cycles would be skipped with aphidicolin did not succeed.

DISCUSSION

Timing of initiation of various events during embryonic development is closely associated to the number of cell divisions rather than elapsed time since fertilization (e.g. Bleyman, 1971; Summerbell, Lewis & Wolpert, 1973; Smith & McLaren, 1977). Therefore, the clock mechanism which is determining the time of initiation of cellular differentiation is likely to be regulated by the events of cell cycle. Three possible candidates for the counting mechanism are cytokinesis, nuclear division, and DNA replication. An experimental result that the time of first AChE occurrence in embryos which have been cleavage-arrested with cytochalasin B or with colchicine is about the same as in normal embryos, clearly indicates that the clock is not directly controlled by the events of cytokinesis and of nuclear division (Whittaker, 1973; Satoh, 1979). As noted previously by others (Brachet & Tencer, 1973; Whittaker, 1973), the nucleus in the cell of cytochalasin-arrested embryos divides in good synchrony with that of normal embryos, suggesting that DNA replication in cytochalasin-treated blastomeres takes place in synchrony with that of normal embryos. Colchicine prevents normal divisions in nucleus, but the cells of colchicine-arrested embryos show cycles of nuclear envelope breakdown and nuclear envelope reformation. In addition, the cells continue incorporating [³H]thymidine for at least 3 h. These results imply that the cycle of DNA replication is maintained in the colchicine-treated blastomeres. Recently, Sluder (1979) reported that in sea urchin eggs exposed to colcemid the cell cycle is prolonged in mitosis. In the case of *Ciona* eggs treated with colchicine a delay in the reappearance of the nuclear envelope was observed. But the time of first AChE development in embryos which had been arrested with colchicine in the 32-cell stage was nearly the same as in normal embryos. The delay in repeated cycles of nuclear envelope breakdown and nuclear envelope reformation, therefore, is not so much to cause the delay of AChE occurrence more than 1 h, because we examined the time of first AChE development in the materials fixed at 1 h intervals.

Previous experiments using actinomycin D and puromycin have revealed that AChE is synthesized from the neurula stage onwards and that new RNA synthesis which begins between the early and late gastrula stages is necessary for AChE development (Whittaker, 1973, 1977; Meedel & Whittaker, 1979; Satoh, 1979). Inhibitors of cellular DNA synthesis have the effect in various

ways (see Cozzarelli, 1977, for a review). Aphidicolin causes selective inhibition of the activity of DNA polymerase- α , leading to blockade of DNA synthesis and cell divisions in sea urchin embryos, while aphidicolin does not affect both RNA and protein synthesis (Ikegami *et al.* 1978). As shown in this study, aphidicolin inhibited about 98 % of labelled thymidine incorporation in ascidian embryos. In spite of the inhibition effect, aphidicolin allowed one more cleavage in eggs kept in aphidicolin and stopped divisions thereafter, no matter how soon after a cleavage it is applied. This may presumably be due to an overlap in time of n th mitosis and $n+1$ th S-phase. Inhibitory effect of aphidicolin on RNA synthesis in ascidian embryos was slight. Embryos which had been permanently cleavage-arrested with aphidicolin in the cleavage stages up to the 64-cell stage did not develop AChE activity, while embryos which had been continuously arrested with the drug from the 76-cell stage onwards eventually produced AChE activity. This result strongly suggests that even if, without DNA replication, a certain time had elapsed since fertilization, the cell would not differentiate. In other words, several cycles of DNA replication might be prerequisite for the cellular differentiation. Since aphidicolin does not alter the DNA itself (i.e. the gene itself), these findings also imply that some qualitative changes in the *state* of the gene take place during the cell cycle between the 64-cell and 76-cell stages. As is reported and discussed in the accompanying paper (Sato & Ikegami, 1981), the eighth DNA replication cycle may be *quantal* for AChE development.

It has been suggested that the clock must be determining the time at which the cytoplasmic information for AChE development interacts with genome (Whittaker, 1973). Therefore, the qualitative changes in the state of the gene might include the interaction of the cytoplasmic information with the genome (cf. Wilson, 1925; Davidson, 1976). Although several models of the mechanism involved in the cytoplasmic factors for cellular differentiation (e.g. Davidson & Britten, 1971) or of DNA replication as the clock mechanism (e.g. Holliday & Pugh, 1975) have been proposed, details of both mechanisms are subjects of further studies. At present we may propose the association of the clock mechanism with the cycle of DNA replication, based on the following evidence: (a) timing mechanism is closely associated with the cell cycle, and (b) DNA replication is prerequisite for the histospecific enzyme development, but not cytokinesis and nuclear division.

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