

Cytological and microspectrophotometric analysis of mesodermalized explants of *Triturus* gastrula ectoderm

By SHINICHI NODA¹ AND IZUMI KAWAKAMI¹

*From the Department of Biology, Faculty of Science,
Kyushu University, Japan*

SUMMARY

Using isolated presumptive ectoderm of the newt (*Triturus pyrrhogaster*) embryos as a reactor and extract of rat bone marrow as a mesodermal inductor, changes of cell number and mitotic index of the reactor cells were studied. In early stages of cultivation the increase in cell number in the mesodermalized ectodermal piece was slower than in the non-mesodermalized epidermal piece; but after 24 h it showed abrupt increase and reached a cell population equal to that of the control at 48 h of cultivation. In the experimental series, the mitotic index was 0 at 4 h after the application of the inducing stimulus, but increased precipitously in the next 8 h and reached a level of 4.4 % at 12 h and thereafter decreased gradually. The cell cycle stopped at the S phase and stayed in it for several hours after the application of inductor. A sudden fall in cell number, observed in the mesodermalized epidermal piece between the 4th and the 8th h after the application of inducing stimulus, seems to be attributable to cell death which was brought about by the inducing stimulus. In the histogenetic process phases of repression on mitosis by an inducing stimulus, cell proliferation and non-proliferation seem to succeed each other.

INTRODUCTION

An inductive system in tissue differentiation is composed of inductor and reactor, and the analysis of the system has to be carried out from both sides. Investigations of the mechanisms of primary induction in amphibian embryos, however, have been chiefly concerned with the inductor; and less attention has been paid to the reactor, the presumptive ectoderm of the gastrula. For the further development of this research field, studies on changes in the reacting tissue seems to be an urgent necessity. This report will be concerned with the analysis of the reacting tissue with special reference to its behaviour at the cell level, the study of which is indispensable for an understanding of tissue differentiation.

Though the interpretation is still uncertain, cessation of cell proliferation preceding tissue differentiation has been reported in several tissues; for example, muscle differentiation in the chick (Stockdale & Holtzer, 1961), endochondral

¹ *Authors' address:* Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812, Japan.

osteogenesis in the rat (Young, 1962), pancreatic differentiation in the mouse (Wessells, 1964) and Wolffian lens regeneration in the newt (Eisenberg & Yamada, 1966). On the other hand, cell death was observed in the differentiation of the avian wing in which some cells in the wing-bud behave as if committing suicide (Saunders & Gasseling, 1962). In the presumptive ectoderm, after it had received the inducing stimulus, cell death has also been demonstrated by the cell electrophoresis technique (Ave, Kawakami & Samashima, 1968). In contrast to this, however, it has been demonstrated by direct cell count or by analytical chemical methods that the mitotic index or DNA synthesis in the responding presumptive ectoderm was enhanced by an inducing stimulus of the organizer (Suzuki, 1968) or by a heterologous neural inductor (Imoh, Sasaki, Kawakami & Hayashi, 1972). To verify these data the authors examined the changes in mitotic index and in cell number, and made a microspectrophotometric analysis of the ectoderm which had been canalized for mesodermal differentiation by rat bone-marrow extract.

MATERIALS AND METHODS

(a) *Preparation of inductor*

The inductor was prepared from rat bone marrow (Kawakami, Watanabe, Ave & Ieiri, 1969). The tissue, taken from rat femur, was homogenized with 10 volumes of Holtfreter's solution (pH 7.2), the supernatant obtained by centrifugation at 10000 *g* for 20 min was adjusted to $\text{O.D.}_{280 \text{ nm}} = 2$. The procedure was carried out in the cold at 0 °C.

(b) *Mitotic index and cell population*

Presumptive ectoderm of early gastrula (Stage 12a by Okada & Ichikawa, 1947) of the newt, *Triturus pyrrhogaster*, was used as a reactor in this experiment. As has already been demonstrated by Kawakami, Watanabe, Ave & Ieiri (1969), the bone-marrow extract solution obtained as described above shows a mesoderm-inducing activity when it acts on the ectoderm. Sterilization of the embryos was carried out by treating them successively with 0.06% Chloramine-T (Wako Pure Chemical Industries, Osaka) and 70% ethanol for 8 min and 50 sec, respectively. After decapsulation and removal of vitelline membrane, a strip of presumptive ectoderm (1.0 × 1.5 mm) of the embryo was excised with hair loop and glass needle. An ectodermal strip was punched with a sharp-edged steel tube whose diameter was 0.6 mm to get two small circular pieces of the same size (Fig. 1). As a control, one of the two small pieces of ectoderm was immediately fixed and stained for 10 min by aceto-gentianviolet and squashed on a glass slide under a coverslip and sealed with nail enamel. Another piece was cultured in Holtfreter's solution for 4, 8, 12, 16, 24, 48 or 240 h at 20 °C and prepared as above. In the experimental series, one piece was fixed and prepared immediately, and the partner piece was treated by the bone-

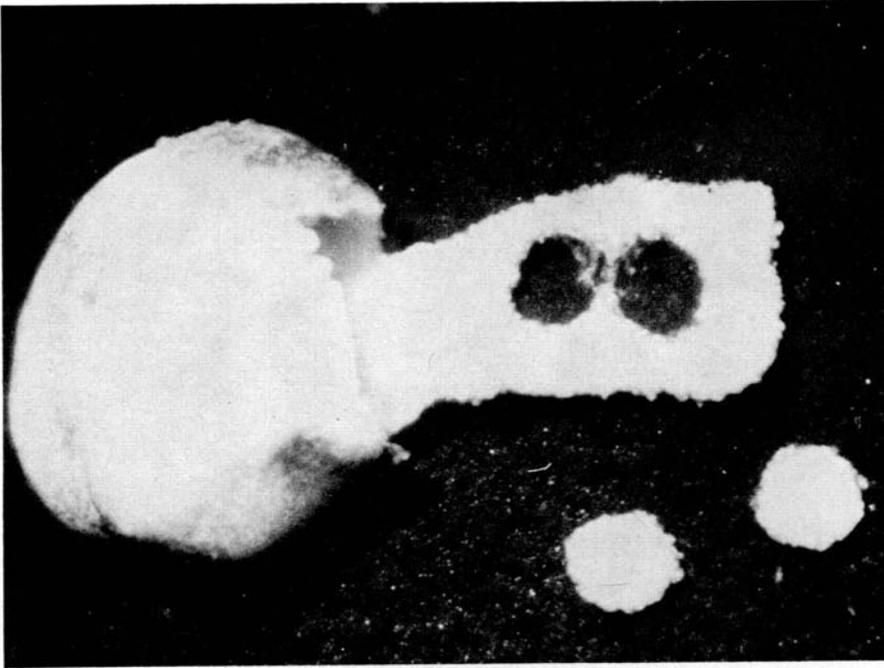


Fig. 1. Two small circular pieces of the same size were cut from presumptive ectoderm by punching with a sharp-edged steel tube. $\times 22$

marrow extract for 3 h at 20 °C for 1, 5, 9, 13, 21, 45 or 237 h. After the cultivation each ectodermal piece was fixed, stained and squashed in the same way as the control. Each slide of the control and the experimental series was carefully scanned under the microscope, and the numbers of mitotic figures (Fig. 2) and cells were counted.

(c) *Microspectrophotometry*

A strip of presumptive ectoderm was prepared as described above. The ectodermal piece was cultured in Holtfreter's solution for 4, 8, 12, 16, 20 or 24 h at 20 °C for the control series. In the experimental series, the ectodermal piece, which was treated by bone-marrow extracts for 3 h at 20 °C, was washed three times with Holtfreter's solution and cultured in the same solution at 20 °C for 1, 5, 9, 13, 17 or 21 h. The piece was then fixed for 10 min in 22% acetic acid. The sample was squashed under a coverslip on a slide, and the coverslip was removed by putting the slide on dry ice. The slide was hydrolysed in 1 N HCl for 12 min at 60 °C and subsequently treated with a mixture of 8.5 volumes Sørensen's 0.1 M glycine buffer (pH 2.28), 1 volume 15% metabisulphate and 0.5 volume Schiff's reagent for 3 h. The slide was successively washed three times with a mixture of 9 volumes Sørensen's 0.1 M glycine buffer (pH 2.28) and 1 volume 15% metabisulphate for 15 min each (Shibatani & Naora, 1952). The Feulgen-stained preparation was washed, dehydrated with an ethanol series,

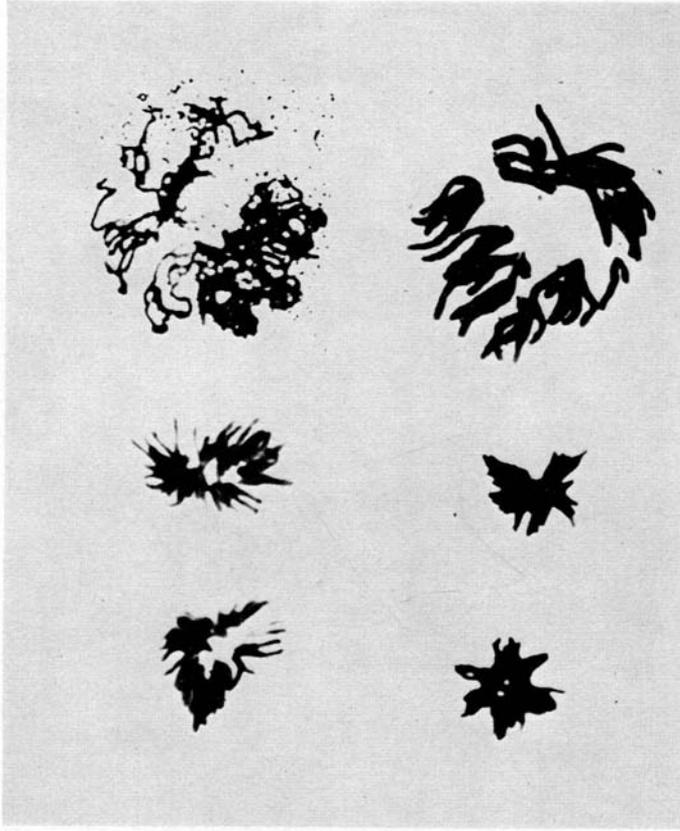


Fig. 2. Showing various phases of mitotic figures, arranged from prophase to teleophase. Nuclei shown in this figure were counted as ones in M phase in this experiment. $\times 1000$

and mounted with Canada Balsam after clearing with xylol. The absorbance of Feulgen-positive materials in individual nuclei was measured by the single wavelength method with the Olympus DMSP-II at 560 nm, and the diameter of individual nuclei was measured at the same time (Pollister, 1955). One hundred interphase nuclei were randomly selected for measurement from each of the squash preparations.

(d) Autoradiography

The cell cycle of the presumptive ectodermal cells isolated from gastrula embryos was determined. To determine the S and G₂ phases, the isolate of presumptive ectoderm was cultured in Holtfreter's solution containing 20 $\mu\text{Ci/ml}$ of [³H]thymidine for 2, 3, 4, 5, 6, 8 or 10 h, and then fixed for 10 min in 22% acetic acid. The fixed presumptive ectoderm was squashed under a coverslip on a slide, and after removal of the coverslip by putting the slides on dry ice, the slides were treated with acid alcohol (1:20) for 5 min. Liquid-sensitive emulsion (Sakura NR-M2, Konishiroku Photo Ind. Co., Ltd) at 45 °C was applied to the

slides in the dark. The slides were exposed for 4 weeks in the cold and then developed with Sakura Konidol X for 5 min, fixed for 10 min in Sakura Konifix, and washed in running water for 30 min. The samples were stained with hematoxylin. The number of silver grains on the chromosomes of each cell was counted under the microscope.

RESULTS

(a) *Changes in mitotic index*

The difference between the mitotic index of the control series and that of the experimental series was significant. The mitotic index of cells in the non-treated ectoderm (control) decreased gradually in the first 48 h from 3.70% to 0.51%, and remained almost unchanged thereafter (0.20%). The change in mitotic index of the mesodermalized explants was more striking than that of the controls in the first 24 h. The mitotic index was 0% at 4 h, and then increased precipitously in the next 8 h to reach a maximum (4.40% at 12 h). After 12 h of cultivation it decreased gradually to 2.97% after 16 h, 1.62% after 24 h and 1.30% after 48 h. Both in the experimental and control series the indexes, however, finally took low values which were 0.12% and 0.20% after 240 h cultivation. The time course of the changes of mitotic indexes in the control and experimental series are presented in Fig. 3 and Table 1.

(b) *Changes of cell number*

In the control series, the cell number of the isolated presumptive ectoderm increased continually. The value was twice as high as the original one at 15 h of cultivation, and the number was nearly tripled at 22 h and quintupled at 240 h. The generation time was deduced by the following formula: $\text{Generation time} = (B - A)(a + 100)/(b - a)$, where A and B are hours of cultivation, a and b are the rates of increase of cell numbers at A and B h of cultivation. The generation time of cells, calculated in this fashion, was lengthened with increase of the cultivation period, i.e. 14.6 h in the cells of explants cultured from 0 to 12 h, 15.4 h in those cultured from 12 to 24 h, 150 h in those cultures for from 24 to 48 h, and 685 h in those cultured from 48 to 240 h. Thus a remarkable prolongation of generation time in the isolated presumptive ectodermal cells took place after 24 h of cultivation.

In the experimental series, cell number increased 11.89% in the first 4 h, but decreased 8.85% over the following 4 h, and again increased steadily after 8 h. The cell number was doubled at 20 h, tripled at 40 h, and quintupled at 240 h. The generation time of the cells of the explants treated with the bone-marrow extract was 63.2 h in those cultured from 0 to 12 h, 12.9 h in those cultured from 12 to 24 h, 46.1 h in those cultured from 24 to 48 h, and 447 h in those cultured from 48 to 240 h. The generation time of treated cells, thus, was about four times as long as that of the control series for the first 12 h, but it became remarkably shorter in the next 12 h, and was about one-third of that in

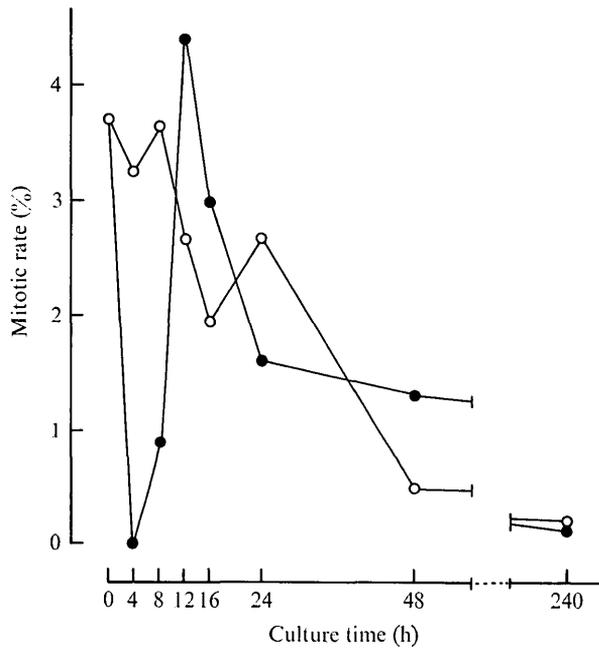


Fig. 3. The time course of changes of mitotic index in ectodermal pieces after responding to the induction stimulus. Each rate is an average obtained from five pieces. O, Control ectoderm; ●, experimental ectoderm.

Table 1. *The mitotic rate and increase of cell number in control and experimental series (each figure is an average obtained from five pieces)*

	Culture time (hours)	Average value \pm standard deviation	
		Control	Treated with B.M. solution
Mitotic rate (%)	0	3.70 \pm 1.70	3.70 \pm 1.70
	4	3.25 \pm 1.08	0.00 \pm 0.00
	8	3.64 \pm 1.93	0.89 \pm 1.02
	12	2.67 \pm 0.60	4.40 \pm 1.56
	16	1.96 \pm 0.56	2.97 \pm 0.96
	24	2.69 \pm 0.41	1.62 \pm 0.72
	48	0.51 \pm 0.08	1.30 \pm 0.56
	240	0.20 \pm 0.12	0.12 \pm 0.15
Increase rate of cell number (%)	4	32.90 \pm 16.55	11.89 \pm 7.92
	8	39.67 \pm 27.38	3.04 \pm 17.27
	12	81.85 \pm 17.86	19.09 \pm 17.18
	16	108.89 \pm 52.21	65.10 \pm 45.21
	24	224.92 \pm 74.68	129.83 \pm 42.56
	48	276.63 \pm 89.64	249.28 \pm 130.72
	240	382.85 \pm 125.46	399.82 \pm 78.81

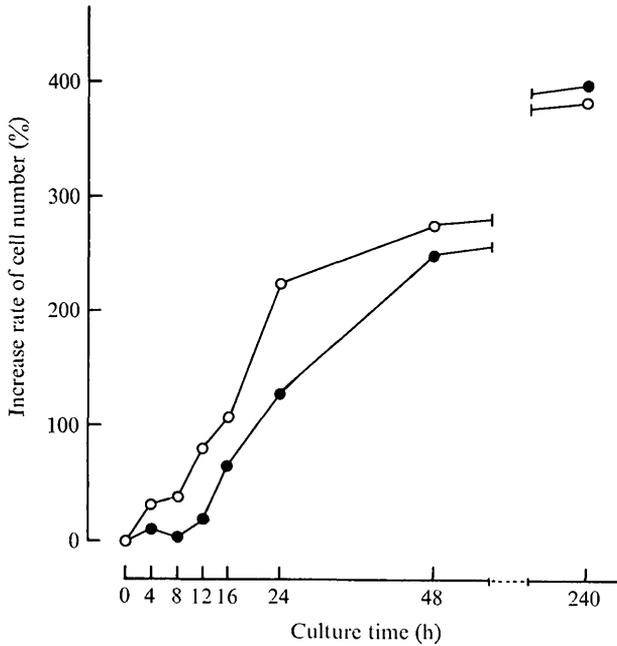


Fig. 4. The time course of changes in cell number in ectodermal pieces after responding to the induction stimulus. Each rate is an average obtained from five pieces. ○, Control ectoderm; ●, experimental ectoderm.

the control series for the following 24 h. Finally, however, the generation time became close to that of the control series after 48 h. Increase of cells in the experimental series was smaller than that in the controls in the early period of cultivation, but it became almost equal to that of the control series after 48 h. The time courses of changes in cell number in the control and experimental series are presented in Fig. 4 and Table 1.

(c) *Microspectrophotometric observations*

The histograms of Fig. 5 and Fig. 6 show the relative values of DNA content of nuclei at various periods of cultivation of presumptive ectoderm and of mesodermalized ectoderm. Each histogram gives the total amount obtained from 400 nuclei. In non-cultured pieces, the relative DNA content of the nuclei shows a varying value, but nuclei of lower value (G1 or early S phase nuclei) are more prominent than those of higher value (late S or G2 phase nuclei). In the control series, the histogram shows a common pattern at each period of cultivation, similar to that of non-cultured pieces. In the experimental series, nuclei with lower DNA content decrease and those with higher DNA content increase gradually in the pieces cultured for 4, 8 and 12 h, but nuclei with lower DNA content increase and those with higher DNA content decrease gradually in the pieces cultured for 16, 20 and 24 h. The histogram of the relative DNA content of nuclei in the pieces cultured for 24 h becomes similar to that in non-cultured

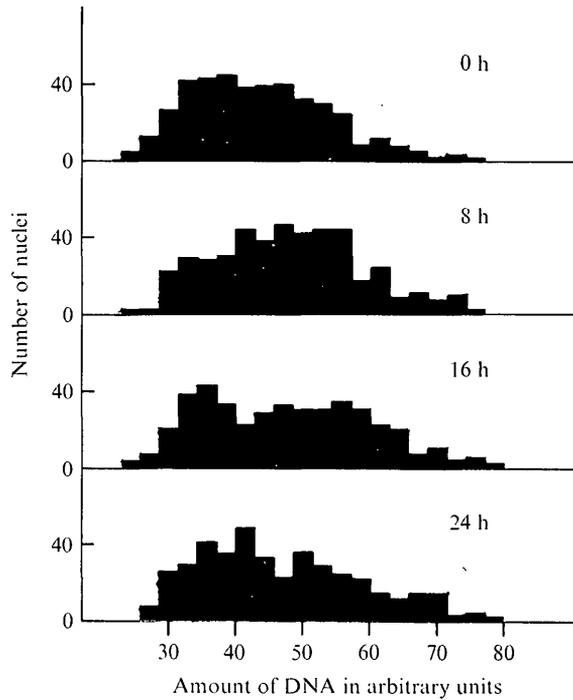


Fig. 5. Histograms of the relative DNA content in nuclei of non-treated ectoderm at various periods of cultivation. The histograms show a common pattern at each period of cultivation, which is similar to that of non-cultured pieces.

pieces. Thus it may be said that the population of S phase cells increases in presumptive ectoderm in the first 12 h after it responds to the mesodermalizing stimulus.

(d) *Determination of cell cycle in isolated presumptive ectoderm*

As mentioned above, the cell number of the ectoderm became twice the original one after 16 h of cultivation. The generation time of ectodermal cell therefore must be about 16 h at least under the present conditions (Fig. 4). The M phase was about 0.5 h which was calculated from the mitotic index of 3.7% (the M phase was calculated from the following formula: $M = T \times a/100$, where M is M phase, T is generation time = 16 h, a is mitotic index = 3.7%) at 0 h. The S and G2 phases were determined by continuous labelling of ectodermal piece by [^3H]thymidine. The average number of silver grains per cell increased gradually, for example 32 at the 2nd hour, 61 at the 3rd hour, 94 at the 4th hour, 127 at the 5th hour, 150 at the 6th hour, 193 at the 8th hour and 196 at the 10th hour (Fig. 7). Because in the G2 phase no [^3H]thymidine is incorporated into the nucleus, the first period of cultivation, during which no silver grains are observed in mitotic figures, corresponds to the G2 phase; and this period was 1 h. The period during which the number of silver grain increases corresponds to the S phase and this was 8 h. Thus, the duration of each phase

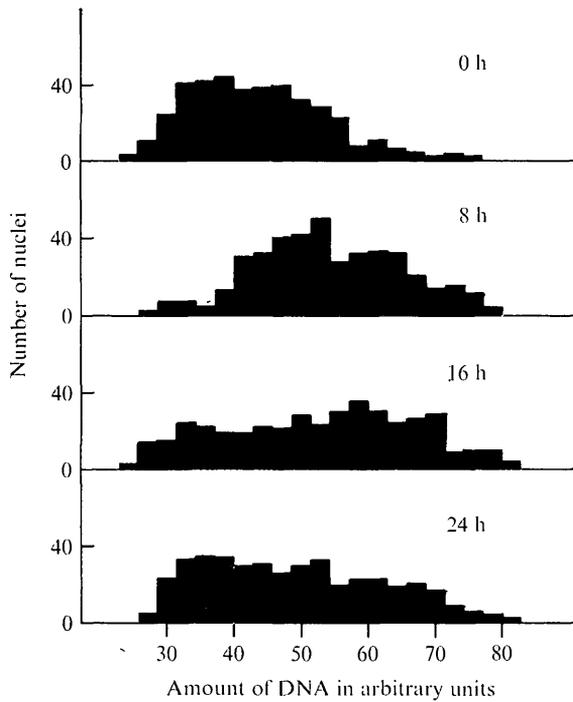


Fig. 6. Histograms of the relative DNA content at various periods of cultivation in nuclei of ectoderm stimulated by mesodermal inductor. The number of nuclei with lower DNA content decreases and that of nuclei with higher DNA content increases in the pieces cultured for 8 h, but the number of nuclei with lower DNA content increases and that of nuclei with higher DNA content decreases in the pieces cultured for 16 h and 24 h.

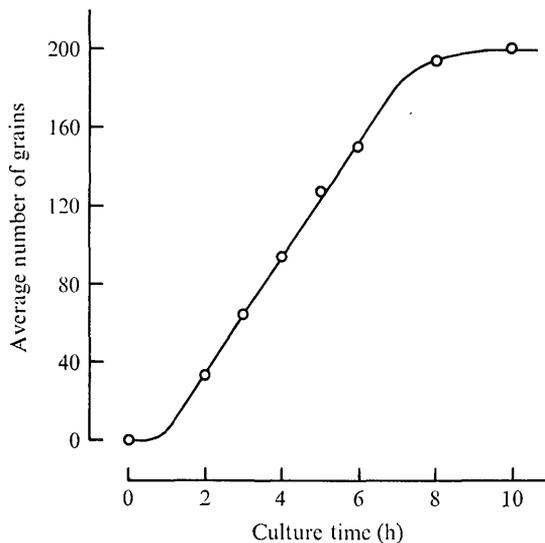


Fig. 7. The increase of average number of silver grains in an ectodermal piece; obtained by autoradiography after continuous labelling with [^3H]thymidine.

of the cell cycle of the ectoderm was determined as 6.5, 8, 1 and 0.5 h for G1, S, G2 and M, respectively. G1 was calculated by subtracting the sum of G2, S and M from the total generation time.

DISCUSSION

The mitotic index of cells in the presumptive ectodermal pieces cultured in Holtfreter's solution decreased gradually during the first 48 h, and remained almost unchanged thereafter. In contrast to the control series, the mitotic index of the mesodermalized ectoderm showed a distinctive change during the first 24 h. It took the value 0 at 4 h, and increased precipitously in the next 8 h to be 4.40% at 12 h, after which the mitotic index decreased again to an almost constant value at the 10th day of cultivation. The difference in the pattern of mitotic indexes between the control and neuralized ectoderms has also been observed by Suzuki (1968), Imoh, Sasaki, Kawakami & Hayashi (1972), and Suzuki & Kuwabara (1974) in *T. pyrrogaster* ectoderm. Suzuki (1968) studied the change in mitotic index of the isolated presumptive neuroectoderm during gastrulation. According to him, the mitotic index of the control ectoderm (not underlain by organizer) was constantly low for 52 h after isolation, while that of the ectodermal piece stimulated by the organizer increased from 6 to 15 h of isolation and decreased thereafter. Imoh, Sasaki, Kawakami & Hayashi (1972) studied the change of the pattern of synthesis of macromolecules in the isolated presumptive ectoderm neuralized by rat liver extract. According to them, in the non-treated ectodermal piece DNA synthesis continued at an almost constant rate for at least 3 days after isolation, but the neuralized ectoderm showed a sharp increase in DNA synthesis up to the 12th hour of inductive treatment and then fell to a lower level than that of the control. A temporary cessation of mitosis and DNA synthesis in the isolated presumptive ectoderm was not observed in this work. Suzuki & Kuwabara (1974) observed a temporary suppression of mitosis in isolated presumptive ectoderm. According to them, the mitotic index of control ectoderm decreased continually for 96 h of cultivation, while that of the isolated ectoderm stimulated by guinea-pig liver extract decreased to 0.65% at 6 h, and increased in the next 18 h to 3.15% at 24 h, after which the mitotic index again decreased. In the present experiments the elapsed time between the beginning of the treatment and the moment showing the maximum value of mitotic index was shorter than that expected from the results of Suzuki & Kuwabara (1974). This is probably not only due to differences in the induction stimulus, which was a mesodermal inductor in our study and neural one in their case, but also to differences in the methods of inductor application. The treatment with bone-marrow extract applied in the present work takes shorter time to realize the maximum response to the induction stimulus as compared with that by the sandwich method (Katoh, 1962; Noda, Sasaki & Ieiri, 1972).

The mitotic cycle of the cells of the presumptive ectodermal isolate was determined by the doubling time of the cell number, the labelling of nuclei and the mitotic index. Thus the G1, S, G2 and M phases were 6.5 h, 8 h, 1 h and 0.5 h, respectively. After application of inductor, the cell cycle of reactor cells seems to stop at the S phase and to stay there for several hours, because few mitotic figures were found at 4 h of cultivation but they appeared in the next 4 h. The same result was shown by the microspectrophotometric observation of mesodermalized ectoderm. In the mesodermalized ectoderm cultured for 8 h, nuclei with lower DNA content decreased and those with intermediate DNA content increased (Fig. 6). This shows that the mitosis of the mesodermalized ectodermal cells has stopped for several hours in the S phase. Contrary to the temporary cessation of mitosis in the isolate of the presumptive ectoderm stimulated with the inductive bone-marrow extract, in the non-treated ectodermal piece the mitotic index gradually decreased for 48 h, during which no violent fluctuations of the index were observed. The intact ectoderm, which had been in contact with the chorda-mesoderm for several hours, showed temporarily lower mitotic activity for several hours (Suzuki & Kuwabara, 1974). The marked decrease of mitosis during the first phase of induction could be due to a shock by the inductive effect, and it may be concluded that the repressive effect of the extract on mitotic activity and the increase of the S phase cell population in the reactor ectoderm have a bearing on the onset of the mesodermalizing activity of the ectoderm. In the cases of pancreas (Wessells, 1964), regenerating retina in *Triturus* (Eisenberg & Yamada, 1966) and the *in vitro* differentiation of lens epithelium from chick embryonic lens (Watanabe & Kawakami, 1973) the first phase of differentiation follows a non-proliferative phase. A similar time course was also confirmed in the present material. Thus it may be concluded that a repressive phase in the eliciting period, a proliferative one during the phase of labile determination, and a following non- or less-proliferative phase in the differentiation phase may be a general phenomenon in histogenesis.

The increase in cell number in mesodermalized explants was small compared with the control explants in the early period of cultivation and at 12 h of cultivation the cell number of the mesodermalized epidermis was only 65% of that found in the control epidermis (Fig. 4). It is interesting to note that this value corresponds to that proposed by Ave, Kawakami & Samashima (1968) in their electrophoretic study on the neuralized ectoderm. They reported that the presumptive ectoderm of the *T. pyrrhogaster* gastrula consisted electrophoretically of at least three kinds of cells, i.e. N, M1 and M2, and their population ratio in the ectoderm was 35, 41 and 24%, respectively. They concluded that one cell species (N) was selectively killed by the mesodermal inducing agent, leaving alive two other species which differentiate into mesodermal tissue, and that these two cell species (M1 and M2) were killed by the neuro-inducing agent, whereupon the other surviving cell species differentiate into neural tissue. According to their hypothesis, it may be assumed that 35% of the original cell

population was destroyed by the mesodermal-inducing protein during the period from 0 to 12 h, which would give the reduction of cell number observed in the present experiment. Thus, cell death – although further observations in morphogenesis are required – may be an important factor in explaining the behaviour of ectodermal cells in primary induction in the amphibian embryo.

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