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Developmental Lesions in Amphibian Embryos Induced by Ultraviolet Irradiation of the Fertile Egg

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자외선에 의한 양서류 수정난의 발생 결함

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摘 要

양서류 수정난의 식물반구를 제 1 난할 전에 자외선 조사를 하면 신경관 형성에 독특한 변형을 유발한다. 자외선에 대한 감응도는 수정에서 제 1 난면이 나타나기까지의 2/3시기에 급격히 저하된다. 주사 전자현미경 사진은 자외선을 받은 배아의 표피세포들의 크기가 감소된 것을 보여주고 있다. 그러나 외배엽 교환이식실험은 자외선을 받은 배아의 외배엽이 아직도 완전히 정상적인 신경조직과 표피를 형성할 수 있는 능력을 보유하고 있음을 나타내고 있다. 위의 사실들은 낭배기간중 함입능력의 저하와 일차형성체의 유도능력의 감퇴와 관련시켜 설명하였다.

INTRODUCTION

One of the central problems of contemporary develpmental biology is the question of how the fertilized egg gives rise to the many different kinds of cells of metazoan organisms. This question deals with the primary problems of the control of gene action and its effects during embryonic development. Evidence from molecular and experimental embryology supports the concept that the nuclei of each cell in the embryo contain identical genetic information. Differentiation is believed to be achieved not by unequally distributed chromosomes, but rather by the

biochemical environment of the chromosomes. Thus, the variety of gene expression pattern is established by interactions between embryonic nuclei and regionally distributed morphogenetic components of the egg cytoplasm. These morphogenetic determinants are believed to be produced during oogenesis, and deployed at appropriate stages of embryogenesis (reviewed by Davidson, 1968; Nieuwkoop, 1973). Although various experimental approaches have been employed, including the analysis of maternal effect genes (Briggs and Justus, 1968), biochemical fractionation (Horstadius, 1973), and physical manipulations (Nieuwkoop, 1969), a thorough understanding of the biochemical characteristics and developmental life cycle of a single morphogen is not yet available.

Among the many types of embryos which provide opportunities for a multifaceted approach to the study of the morphogens is the amphibian egg. The opportunities for embryological, biochemical, and genetic analysis of amphibian embryos are well documented (reviewed by Malacinski and Brothers, 1974). In several laboratories various methods for analyzing cytoplasmic localizations in amphibian egg have been employed, including cortical grafting (Curtis, 1962), cytoplasmic transfers (Smith, 1966), and localized ultraviolet irradiation (Grant, 1969). Of these methods, the use of ultraviolet light (UV) to induce specific developmental lesions in the amphibian egg provides perhaps one of the potentially most convenient tools for the analysis of cytoplasmic localizations in amphibian eggs. There exists a backlog of information on the effects of UV on several animal eggs (Baldwin, 1915; Goldman and Setlow, 1956; Arnold, 1968); the mechanics of UV treatment are easily carried out; and the results of preliminary experiments (Grant and Wacaster, 1972) suggest that UV affects specific developmental events in amphibian embryogenesis. That is, UV irradiation of the vegetal hemisphere of the egg destroys the embryo's capacity for normal axis formation and neural morphogenesis. Experiments from our laboratory confirmed that the target was mainly localized in the dorsal side of the vegetal hemisphere (Malacinski *et al.*, 1975).

Although those previous experiments clearly demonstrated that the developmental lesion in UV irradiated (UV'd) eggs is abnormal neural morphogenesis, there has not been any detailed study on the external morphology of the irradiated embryo. In this report, it was possible to reveal fine structural changes in ectoderm cells of irradiated embryo by using an advanced technique of scanning electron microscopic analysis. In order to gain further insight into the UV effect, we also investigated the capacity of the ectoderm cells of the UV'd embryo to undergo normal neural morphogenesis by exchanging this area between UV'd and non-UV'd embryos. The results are discussed in terms of the cellular movements at gastrula stage and primary embryonic induction. The relative

sensitivity of the egg to the UV irradiation was also revealed. We have chosen *Rana nigromaculata*, since it has much faster developmental rate than *Rana pipiens* in early embryogenesis, and a valid conclusion on the critical period of the UV sensitivity could be drawn.

MATERIALS AND METHODS

Source of Amphibian: Northern Amercan leopard frog, *Rana pipiens*, has been used for the most of our previous work and also was used here for the characterization of the general feature of the UV lesions and for the scanning electron microscopy. They were obtained from commerical dealers and maintained in cold room (5°C) until used. *Rana nigromaculata* was used for the study of capacity of the ectoderm to undergo normal neural morphogeneis and for the study of the relative sensitivity to UV irradiation. They were collected in the spring from several areas in South Korea and used immediately or stored in a refrigerator (5°C—10°C) for 1—2 weeks. Both species were routinely induced to ovulate by injection of pituitary glands, and artificial insemination was carried out with a freshly made sperm suspension on a Petri dish (Hamburger, 1960).

UV Irradiation: *Rana pipiens* eggs were manually dejellied and irradiated within 90 minutes after fertilization with approximately 6,000~24,000 ergs/mm^2 of 2537Å UV at the vegetal hemisphere. *Rana nigromaculata* eggs were given high dose of UV (24,000 ergs/mm^2) for both the studies of the ectoderm swap and the relative sensitivity to the irradiation. Batches of about 20 eggs were loaded on a quartz slide in dechlorinated tap water above the UV source. At tail bud stage, the degree of UV lesion was scored as described before (Malacinski *et al.*, 1975, and Results).

Scanning electron microscopic analysis: *Rana pipiens* embryos were fixed in glutaldehyde solution as described by Kalt (1971). Fixed embryos were dried and coated by standard critical-point drying and coating methods (Anderson, 1951), and the dried embryos were placed on platforms (Malacinski *et al.*, 1977).

Microsurgical Operation: The ectoderm exchange followed methods recently described by Hennen (1973) and modified by Chung and Malacinski (1975). The vitelline membrane was manually removed with a pair of watchmaker's forceps and the operation was performed on a 2% agar bottomed Petri dish which contained 100% Steinberg's solution (Steinberg 1957) with antibiotics (pH 7.4, 4 x Ca^{++}, containing 5 mg of penicillin-G and streptomycin sulfate per liter). The ectoderm areas were reciprocally exchanged between irradiated and nonirradiated embryos at the stage of dorsal lip formation (Shumway stage 10). To lower the incidence of exogastrulation, the grafted embryos were changed to 20% Steinberg's

solution containing the above mentioned antibiotics ahout 2~3 hours after the operation.

RESULTS

Irradiation of the vegetal hemisphere of frog eggs before the first cleavage division results in the development of embryos which display a diminished capacity for morphogenesis of anterior axial structure. The amount of UV required to produce lesions in neural development of *Rana pipiens* was determined by both a direct measurement of the output of the UV lamp and a comparison of the amount of UV required to inactivate free sperm, produce sterile embryos, and diminish the capacity for normal neural development. The intensity of UV at the surface of the vegetal hemisphere, was measured to be 100 ergs/sec/mm^2. As little as 50 ergs/mm^2 was sufficient to inactivate more than 95% of the sperm in a fresh suspension as determined by the frequency of the haploid syndrome (Subtelny, 1958) in activated eggs. 9,000 ergs/mm^2 of UV irradiation at the vegetal hemisphere just as the first cleavage furrow formed produced embryos which displayed a normal external morphology but in more than 90% of the cases completely lacked primordial germ cells. Treatment of 10,000 ergs/mm^2 at the vegetal hemisphere at 90 minutes after fertilization was, however, required to produce microcephalic embryos, while approximately 20,000 ergs/mm^2 was required to manifest aneural development.

However, the degree of response to the same dose of UV is variable from batch to batch, even within the same clutch of eggs. That is, if a batch of eggs are irradiated, a gradient of response is usually observed. Hence, we have previously devised the method of categorizing embryos on a scale of 0 (no effect), +1 (microcephaly), +2 (extreme microcephaly), +3 (poor head morphology, and shortened axial structure), +4 (acephalic), and +5 (aneural). The gradient of scale is shown in Fig. 1.

It has already been demonstrated in our earlier works that the relative sensitivity of *Rana pipiens* eggs to UV changes dramatically during the time period

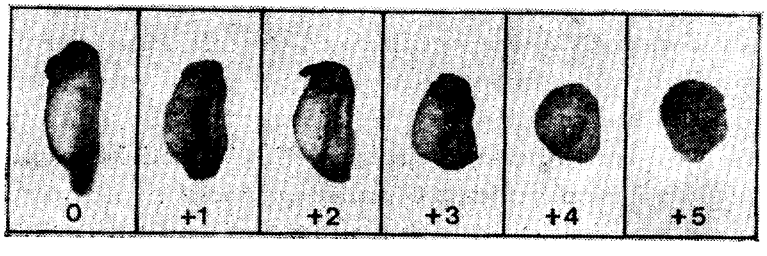

Fig. 1. Irradiated *Rana pipiens* embryos display a typical gradient of response. The extent of damage was scored on a scale from 0 to +5. For explanation, see text.

between fertilization and the first cleavage division. At 22°C, a sharp decrease in the number of eggs which display UV syndrome appears around 90 minutes after fertilization, and the eggs start their first cleavage division at approximately 2.5 hours after fertilization (Malacinski *et al.*, 1975). At the same temperature, *Rana nigromaculata* eggs rotate at approximately 22~25 minutes after fertilization, and begin to appear the first cleavage furrow at 75~80 post-ferti lization. Table 1 shows a typical result of the changing pattern of UV sensitivity, where the number of embryos displaying each degree of the syndrome are shown. At each period, the eggs were irradiated with high dose of UV (24,000 ergs/mm²).

Table. 1. Results of the UV sensitivity of developing *Rana nigromaculata*

UV irrad. after fert.(min)	Abnor. dev.	0	+1	+2	+3	+4	+5	Average UV effect
30							17	5
40							18	5
50	1	1	0	1	2	1	15	4.4
60	1	16	1	2	1			0.3
70	1	19						0
80	1	19						0
90	2	18						0
100	1	19						0
110	1	19						0

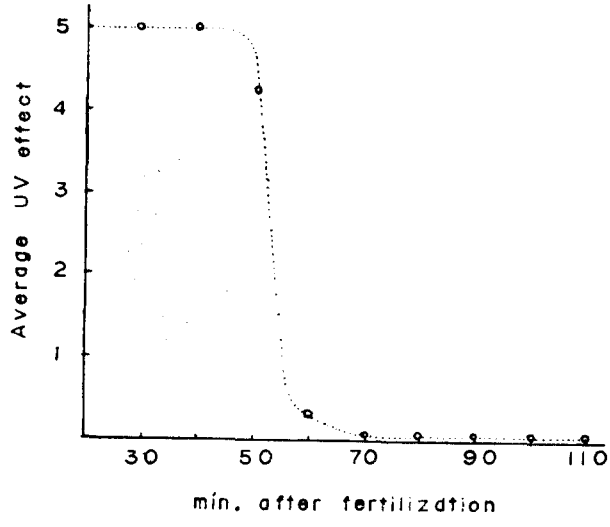

Fig. 2. Relative sensitivity of developing *Rana nigromaculata* to UV irradiation. A batch of 20 eggs was irradiated at each time point and the average effects are plotted on the ordinate. The first cleavage furrows appear approximately 75~80 min after fertilization at 22°C.

In Fig. 2. a plot of the average UV effect at each time is indicated. It is clearly revealed that a drastic drop in sensitivity starts at approximately 50 minutes after fertilization and the change lasts about 10 minutes. After 60 minutes post-fertilization, there is virtually no response to UV irradiation.

Scanning electron micrographs of the anterior portion of embryos which displayed diminished head sizes are shown in Fig. 4. Low UV doses (6,000~12,000 ergs/mm^2) diminished the size of both the head and cement gland (ventral sucker) while higher doses (15,000~18,000 ergs/mm^2) eliminated the cement gland completely. Even higher doses (more than 24,000 ergs/mm^2) destroyed the embryo's capacity to exhibit any of the external features of differentiation of anterior axial structures.

Although the most obvious developmental lesion in UV'd eggs was abnormal neural morphogenesis, during the course of the scanning electron microscopic analysis of anterior axial structures it was observed that ciliated cells appeared to be more densely arranged over the surface of irradiated embryos than on control embryos of comparable ages. Fig. 5 displays both control and UV'd embryos and details of their ventral-lateral surfaces showing the arrangement of ciliated cells. A comparison of Figs. 5b and d reveals that at stage 17 (tail bud stage) the surface epidermal cells of UV'd embryos are somewhat smaller in size than those of control embryos. The length of the cilia is also shorter on UV'd embryos than on control, unirradiated embryos of the same age.

At stage 21 (mouth open and cornea transparent) a similar pattern was observed (Figs. 5g and h). Although the cilia on the UV'd embryos appeared to be approximately the same length as on control embryos, the size of the cells on the surface of UV'd embryos was substantially smaller than on control embryos. These results indicate, therefore, that irradiation of the egg has effects not only on neural induction, but at later stages on cell growth and cilia differentiation as well (see Discussion).

The influence of the UV irradiation on the capacity of the ectoderm in neural morphogenesis was examined by directly exchanging the ectoderm areas between

Table 2. Results of the presumptive ectoderm exchange between irradiated and unirradiated embryos

Expt.	0	+1	+2	+3	+4	+5
Control (4 min UV after 50 min post-fert.)					21	
UV'd→non UV'd	16					
non UV'd→UV'd					16	
non UV'd→non UV'd	20					

*UV'd : UV irradiated.

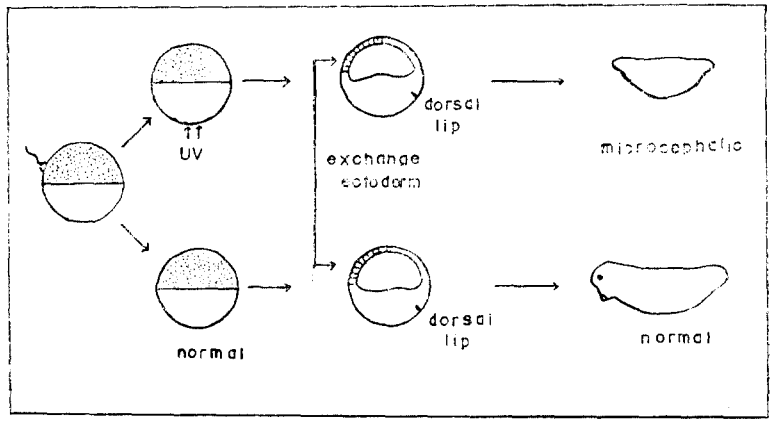

Fig. 3. Experimental procedure for ectoderm exchange. At early gastrula stage the future ectoderm area was reciprocally exchanged between normal and irradiated embryos. At tail bud stage the average UV effects was scored.

irradiated and unirradiated normal control embryos. The design of the experimental procedures is given in Fig. 3. The embryos which had been irradiated with high dose (24,000 ergs/mm^2) displayed 3 hours delay in dorsal lip formation. Only embryos having same size and shape of dorsal lip were selected for the operation. The data in Table 2 indicate that all of the unirradiated embryos which were grafted with ectoderm from irradiated embryos displayed completely normal neural morphology. On the contrary, all of the irradiated embryos received ectoderm from non-UV'd embryos showed the same extent of defects as unoperated UV'd embryos. In other words, the presence of normal ectoderm was not able to correct the developmental lesion induced by UV irradiation, and the presence of ectoderm from irradiated embryo did not cause any UV syndrome in unirradiated embryo.

DISCUSSION

The most characteristic feature of UV'd embryos was their microcephalic character. As the dose of UV was increased from low to high the size of the anterior neural structures was progressively diminished, then the head (including the cement gland) was completely absent. For example, low doses usually produce a milder syndrome (0 to +3), while high doses manifest more severe restrictions on neural morphogenesis including a complete destruction of the embryo's capacity to neurulate (+4 to +5). Thus, the effects are proportional to the amount of UV irradiation.

The phenomenon of the UV effects on normal axial structures was consistently

demonstrated from all batches of different species of amphibians including *Xenopus laevis*, *Bombina orientalis*, and the Mexican axolotl-*Ambystoma mexicanum*. Examination of histological cross-sections of the irradiated embryo revealed that the size of the neural tube was most drastically affected, and that the notochord was, as well, frequently diminished in size. Experiments on the localization of the UV target have shown that the target is mainly on the future dorsal side of the egg. The gray crescent is also located on the future dorsal side of the egg (Malacinski *et al.*, 1975). The effects of UV irradiation on subsequent neural morphogenesis can be corrected by 3 distinctly different methods. These include dorsal lip grafts, low temperature treatment, and microinjection of oocyte homogenate (Malacinski *et al.*, 1974 ; Chung and Malacinski, 1975).

To establish a critical period of sensitivity to UV irradiation it is necessary to compare the changing pattern in various species which have different developmental rates. For this purpose, we selected *Rana nigromaculata* whose egg shows a much faster devolopmental rate than *Rana pipiens* in early embryogenesis. As indicated in Table 1 and Fig. 2, all of the eggs which were irradiated with a high dose before 40 minutes post-fertilization showed a severe lesion in neural morphology (aneural), while all of the eggs irradiated after 70 minutes post-fertilization were completely insenstive (normal). Amazingly, an abrupt decrease occurred within a 10 minutes period. The first cleavage furrow appeared about 75—80 minutes after fertilization. As demonstrated in earlier paper, the *Rana pipiens* egg which has a slower developmental rate, shows a sharp decrease around 90 minutes after fertilization and the egg starts its first cleavage division at about 2.5 hours after fertilization. Both species, therefore, tell us that a critical point of the UV sensitivity is around the time of the 2/3 point between fertilization and first cleavage division.

Considering that *Rana pipiens* eggs show a variable response to UV irradiation even at the time of the first cleavage division, *Rana nigromaculata* eggs display a more uniform response. Furthermore, the *Rana nigromaculata* egg exhibits a clear sperm pit. Usually the egg is monospermic. The sperm pit appears approximately 30 minutes after fertilization, and it lasts until the first cleavage division. It is quite helpful to locate the future dorsal side of the egg since, in most cases, the gray crescent is very hard to detect. The position of sperm entrance and that of future dorsal lip appear on opposite sides of the egg (unpublished observation). *Rana nigromaculata* should be, therefore, considered as a better experimental system in these regards. However, the mechanism of changing sensitivity is not known. One possibility is a reduction in UV sensitivity of the cortex or membrane itself. The other possibility is a displacement of the sensitive cytoplasmic component(s) toward the egg interior as it prepares for first cleavage (Grant, 1969).

Scanning electron micrographs indicate that the epidermal cells of UV'd embryos are smaller in size at tail bud stage. The length of cilia is also affected at this stage but appear to be normal at a later stage (hatching stage). Since cilia appear about the time the neural folds close (Twitty, 1928), the UV effects on cell growth and cilia differentiation seem to be a secondary or at least not primary effect. In addition, both UV'd and non-UV'd embryos showed same number of ciliated cells (unpublished observation). Also, the eggs which received high dose of UV in the vegetal hemisphere displayed a delay in dorsal lip formation, and displayed a wrinkled ectoderm during gastrulation. All the above facts strongly suggest to us that the size of the ectoderm cells and the length of cilia are decreased not by a direct effect of UV but by a failure to stretch to cover the surface of the embryo due to a poor morphogenetic movement of the dorsal lip during gastrula stage.

However, the results of the ectoderm swaps clearly demonstrated that the ectoderm of UV'd embryo are quite normal in its capacity to form a normal neural morphology and epidermis when it is grafted on a non-irradiated embryo at stage 10 (dorsal lip formation). Considering together this result with our recent observation (Malacinski *et al.*, 1977), a decrease in the capacity for invagination and a diminution in the neural inducing capacity of the primary organizer are sufficient to account for the defects in neural development of irradiated embryo.

SUMMARY

Ultraviolet irradiation of the vegetal hemisphere of the fertilized frog egg prior to first cleavage resulted in alterations in neural morphogenesis. The sensitivity to UV irradiation dropped drastically at the point of 2/3 time lapse between fertilization and the appearance of the first cleavage furrow. Scannining electron micrographs revealed a decrease in size of the cells on the surface of the irradiated embryos. However, ectoderm exchange grafts indicated that the ectoderm of the irradiated embryo retains its capaciiy to form a completely normal neural morphology and epidermal surface. These facts were interpreted in terms of a decrease in the capacity for invagination during gastrulation, and subsequent primary embryonic induction.

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REFERENCES

Anderson, T.F., 1951. Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. *Trans. N.Y. Acad. Sci. Ser. II.* **13** : 130—134.

Arnold, J.M., 1968. The role of the egg cortex in early cephalopod development. *Develop. Biol.* **18** : 180—197.

Baldwin, W.M., 1915. The action of ultra-violet rays upon the frog's egg. I. The artificial production of spina bifida. *Anat. Record.* **9** : 365—381.

Briggs, R., and J.T. Justus, 1968. Partial charaterization of the component from normal eggs which corrects the maternal effects of gene *o* in the Mexican axolotl (*Ambystoma mexicanum*). *J. Exp. Zool.* **167** : 105—116.

Chung, Hae-Moon and G.M. Malacinski, 1975. Repair of ultraviolet irradiation damage to a cytoplasmic component required for neural induction in the amphibian egg. *Proc. Nat. Acad. Sci. U.S.A.* **72** : 1235—1239.

Curtis, A.S.G., 1962. Morphogenetic interactions before gastrulation in the amphibian *Xenopus laevis*—the cortical field. *J. Embryol. Exp. Morphol.* **10** : 410—422.

Davidson, E.H., 1968. Gene activity in early development. Academic Press, New York.

Goldman, A.S., and R.B. Setlow. 1956. The effects of monochromatic ultraviolet light on the egg of Drosophila. *Exptl. Cell Res.* **11** : 146—159.

Grant, P., 1969. Nucleo-cortical interactions during amphibian development. *In*: Biology of Amphibian Tumors (edited by M. Mizell). Springer-Verlag, Baltimore, pp. 43—51.

Grant, P. and J.F. Wacaster, 1972. The amphibian gray crescent region—a site of developmental information? *Develop. Biol.* **28** : 454—471.

Hamburger, V., 1960. A Manual of Experimental Embryology. The University of Chicago Press.

Hennen, S., 1973. Competence tests of early amphibian gastrula tissue containing nuclei of one species (*Rana palustris*) and cytoplasm of another (*Rana pipiens*). *J. Embryol. Exp. Morphol.* **29** : 529—538.

Horstadius, S., 1973. Experimental Embryology of Echinoderms. Clarendon Press, Oxford.

Kalt, M.R., 1971. Improved preservation of amphibian embryos for electron microscopy. *Anat. Res.* **169** : 352.

Malacinski, G.M., C.D. Allis, and H-M. Chung, 1974. Correction of developmental abnormalities resulting from localized ultraviolet irradiation of an amphibian egg. *J. Exp. Zool.* **189** : 249—254.

Malacinski, G.M., H. Benford, and H-M. Chung, 1975. Association of an ultraviolet irradiation sensitive cytoplasmic localization with the future dorsal side of the amphibian egg. *J. Exp. Zool.* **191** : 97—110.

Malacinski, G.M., and A.J. Brothers, 1974. Mutant genes in the Mexican axolotl. *Science* **184** : 1142—1147.

Malacinski, G.M., A.J. Brothers, and H-M. Chung, 1977. Destruction of components of the

neural induction system of the amphibian egg with ultraviolet irradiation. *Develop. Biol.* **56** : 24—39.

Nieuwkoop, P.D., 1969. The formation of the mesoderm in urodelean amphibians. II. The origin of the dorso-ventral polarity of the mesoderm. *Wil. Roux' Archiv.* **163** : 298—315.

Nieuwkoop, P.D., 1973. The "organization center" of the amphibian embryo: its origin, spatial organization, and morphogenetic action. *Adv. Morph.* **10** : 1~39.

Smith, L.D., 1966. Role of a "germ plasm" in the formation of primordial germ cells in *Rana pipiens. Develop. Biol.* **14** : 330~347.

Steinberg, M., 1957. Carnegie Inst. Washington Year Book, 56 : 347 (reported by J.D. Ebert)

Subtelny, S., 1958. The development of haploid and homozygous diploid frog embryos obtained from transplantations of haploid nuclei. *J. Exp. Zool.* **139** : 263—298.

Twitty, V.C., 1928. Experimental studies on the ciliary action of amphibian embryos. *J. Exp. Zool.* **50** : 319—344.

ILLUSTRATIONS OF FIGURES

Fig. 4. Scanning electron micrographs (60X) of irradiated *Rana pipiens* embryos show the effects of increased doses of UV on external features of anterior axial structures. (a) control, unirradiated embryc; (b) 6,000 ergs/mm^2 resulted in diminished head size and ventral sucker; (c) 9,000 ergs/mm^2 eliminated ventral sucker; (d) 12,000 ergs/mm^2 drastically reduced size of head; (e) 18,000 ergs/mm^2 almost eliminated external features of head.

Fig. 5. Scanning electron micrographs of normal and irradiated embryos show the effects of increased doses of UV on the development of cilia. (a) normal stage 17 embryos (20X) and (b) detailed view of ciliated epidermal cells (1,200X). (c) UV'd embryo of same age as (a)—20X and (d) detailed view of ciliated cells showing both smaller cell size and apparent shorter length of cilia. (e) normal stage 21 embryo (15X) and (f) detailed view of ciliated cells. (g) UV'd embryo of same age as (e) shows denser packing of ciliated cells and (h) detailed view of ciliated cells showing approximately the same length as on control embryo.

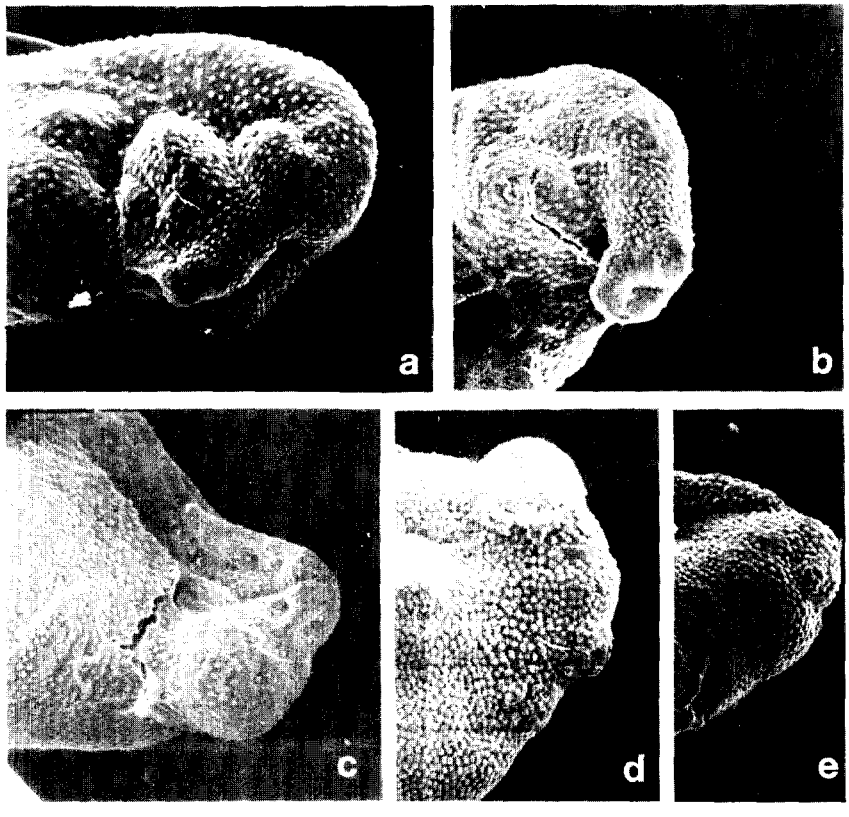

Fig. 4.

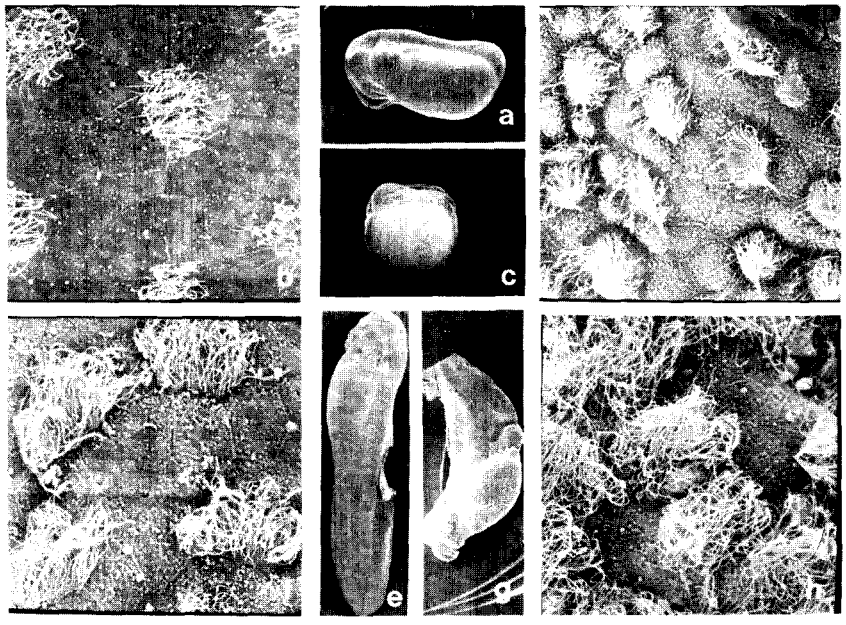

Fig. 5.