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Effects of Ultraviolet Rays on the Sperm of *Rana japonica*

I. Production of Gynogenetic Haploids

By

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(With 7 Text-figures)

INTRODUCTION

It has been well known since 70 years ago that irradiation of sperm is very effective in producing gynogenetic haploids. O. HERTWIG (1911) obtained haploids principally in the European brown frog, *Rana fusca* (=*temporaria*), by inseminating eggs with sperm exposed to radium bromide. RUGH (1939), RUGH and EXNER (1940) and BRIGGS, GREEN and KING (1951) also obtained many gynogenetic haploids in the American leopard frog, *Rana pipiens,* by inseminating eggs with sperm heavily irradiated with X-rays. The finding that UV-rays are very effective in producing gynogenetic haploids was first elucidated by SIMON (1930) and DALCQ (1930) in European brown frogs. SELMAN (1958) also produced gynogenetic haploids by irradiating sperm with UV-rays in three species of European newts, *Triturus*.

On the other hand, the method for incapacitating the sperm nucleus with toluidine blue was devised by BRIGGS, GREEN and KING (1951). According to their study, the majority of the *Rana pipiens* eggs inseminated with *Rana pipiens* or *Rana catesbeiana* sperm which had been exposed to this dye were typical haploids. By applying this toluidine blue method to the Japanese pond frog, *Rana nigromaculata,* MIYADA (1960) obtained abundant gynogenetic haploids and succeeded in raising some of them beyond metamorphosis.

In 1967, some experiments were carried out in our laboratory to establish the best method of incapacitating sperm nuclei in the Japanese brown frog, *Rana japonica,* by the toluidine blue or UV method in order to produce gynogenetic haploids with ease and high probability. Of the results obtained from these experiments, the production of gynogenetic haploids by the toluidine blue method was reported by NISHIOKA and KONDO (1978). They found that the eggs inseminated with sperm treated with 0.005% toluidine blue solution for 40 or 60 minutes cleaved normally and nearly all the normally cleaved eggs became typical gynogenetic haploids.

POGANY (1971, 1973, 1976) examined the HERTWIG effect by irradiating sperm with UV-rays in *Rana pipiens* and found that low-dose embryos were severely

affected in spite of their diploid condition. This finding was in contrast to that obtained in our laboratory in 1967 by the study on production of gynogenetic haploids by means of UV-irradiated sperm. In order to confirm the results obtained in 1967, further experiments were performed in our laboratory in 1977. The results obtained during these two years will be reported here as Parts 1 and 2.

MATERIALS AND METHODS

Males and females of the Japanese brown frog, *Rana japonica* GÜNTHER, were collected in the suburbs of Hiroshima. The females began to ovulate naturally in February and March of the following year. Fertilization capacity of the eggs of each female was examined two days after the beginning of ovulation by using a small number of eggs. Twenty females were selected from those in which more than 95% of the test eggs began to develop normally and were utilized for producing gynogenetic haploids. The UV source was a U-shaped mercury-vapor lamp (GUL-5·J Type, Toshiba Electric Company, Tokyo). This lamp was placed in a metallic casing and operated under a current of 125 mA at 54 V. The UV generated was 2537 Å in main wave length. Sperm were obtained from 20 males which had been kept in the laboratory since fall of the previous year. Sperm suspension was made by crushing the testes of each male into small pieces in 20 ml of tap water. After 0.5 ml of sperm suspension was poured into a vial which was 2 cm in both diameter and height and had a flat bottom, the vial was placed 20 cm from the U-shaped lamp and irradiated for eight or ten different durations, that is, 2.5, 5, 10, 15, 20, 30, 40 and 60 seconds or 80 and 100 seconds in addition at 24 erg/mm^2/sec.

While the eggs of each of 13 females (Nos. 1~13) were divided into 11 parts, those of each of the remaining seven females (Nos. 14~20) were divided into nine parts. One of the nine or 11 parts was inseminated with non-treated sperm as control, while the other parts were inseminated with sperm irradiated for eight or ten different durations. Each part of the eggs was reared at 17±0.5°C until the hatching stage in a small glass dish which was 7 cm in diameter and 6 cm in height. The hatched tadpoles were reared until completion of metamorphosis under laboratory conditions (14~20°C) except those derived from sperm irradiated for 80 or 100 seconds.

Chromosomes were examined in individuals which were living when the control embryos arrived at the hatching stage. Preparations were made from their tail-tips and heads by the squash method with water pretreatment described by MAKINO and NISHIMURA (1952). In some normally hatched tadpoles, two tail-tips, the original and the newly regenerated, were removed from each individual and used for chromosome observation without colchicine pretreatment. These tadpoles were continuously reared after removal of their tail-tips. The other tadpoles and embryos were pretreated with 0.05% colchicine solution for 16~18 hours before squash preparations were made. The haploidy was usually determined by examining chromosome number and nuclear size. However, in

the series consisting of numerous tadpoles, haploids were identified on the basis of their typical haploid characters in appearance. In all the tadpoles other than typical haploids and normal diploids in appearance, chromosome number and nuclear size were examined in order to determine whether they were haploids or not.

OBSERVATION

I. Control series

Twenty matings (Nos. 1~20) were made between 20 females (Nos. 1~20) and 20 males (Nos. 1~20). In each mating, 80.4~100% of the respective number of eggs cleaved normally. In total, 1532 (94.5%) of 1621 eggs did so. Of the normally cleaved eggs, 60 died of abnormalities before the hatching stage and 17 hatched and became abnormal tadpoles, while the other 1455 eggs (95.0%) became normally shaped tadpoles. In each of the 20 series, 90.0~100% of the normally cleaved eggs were normal at the hatching stage. There were no embryos with typical haploid appearance. During the tadpole stage 87 individuals died of ill-development, edema and some other abnormalities. Of the normally cleaved eggs, 75.6~97.7% in each mating, 1368 eggs (89.3%) in total, became normally metamorphosed frogs (Table 1; Fig. 1).

The 17 individuals which were abnormal in appearance at the hatching stage were examined in terms of chromosome number and nuclear size. Preparations were made from their tail-tips and heads by the squash method. These abnormal individuals were all found to be diploids, that is, 26 in chromosome number and there were no haploids among them (Table 2).

II. Experimental series

1. Development of eggs inseminated with irradiated sperm

a. Irradiation for 2.5 seconds

A total of 2057 eggs obtained from 20 females were inseminated in 20 matings (Nos. 1~20) with sperm which had been obtained from 20 males and irradiated for 2.5 seconds.

It was found that a total of 1928 (93.7%) eggs, 71.6~99.3% of the respective number of eggs in each mating, cleaved normally. Of the normally cleaved eggs, 60, 149 and 357 died of various abnormalities at the gastrula, neurula and tail-bud stage, respectively. A total of 859 (44.6%) of the normally cleaved eggs, 15.1~77.9% in each mating, were normal tadpoles at the hatching stage, while a total of 503 were abnormal (Fig. 2a). After 266 of the normally hatched tadpoles died of ill-development, edema or some other abnormalities, the remaining 593 (30.8%) tadpoles, 11.0~48.8% in each mating, metamorphosed normally (Table 1; Fig. 1).

TABLE 1
Development of eggs inseminated

Dose of UV (sec.)	No. of eggs	No. of normally cleaved eggs	No. of gastrulae		No. of neurulae	
			Normal	Abnormal	Normal	Abnormal
0 (Control)	1621	1532	1512 (98.7%)	20 (1.3%)	1503 (98.1%)	9 (0.6%)
2.5	2057	1928	1868 (96.9%)	60 (3.1%)	1719 (89.2%)	149 (7.7%)
5	2265	2125	1977 (93.0%)	148 (7.0%)	1605 (75.5%)	372 (17.5%)
10	2318	2114	1858 (87.9%)	256 (12.1%)	1233 (58.3%)	625 (29.6%)
15	2015	1814	1649 (90.9%)	165 (9.1%)	1081 (59.6%)	568 (31.3%)
20	2072	1881	1777 (94.5%)	104 (5.5%)	1449 (77.0%)	328 (17.4%)
30	2081	1959	1871 (95.5%)	88 (4.5%)	1613 (82.3%)	258 (13.2%)
40	2020	1902	1857 (97.6%)	45 (2.4%)	1731 (91.0%)	126 (6.6%)
60	1935	1840	1821 (99.0%)	19 (1.0%)	1767 (96.0%)	54 (2.9%)
80	716	645	625 (96.9%)	20 (3.1%)	583 (90.4%)	42 (6.5%)
100	823	723	711 (98.3%)	12 (1.7%)	696 (96.3%)	15 (2.1%)

TABLE 2
Normally and abnormally shaped embryos at the hatching stage

Dose of UV (sec.)	No. of eggs	No. of normally cleaved eggs	No. of embryos at				
			Total no.	Percentage to normally cleaved eggs	Normally shaped		
					No.	Percentage to normally cleaved eggs	Percentage to hatching embryos
0 (Control)	1621	1532 (94.5%)	1472	96.1	1455	95.0	98.8
2.5	2057	1928 (93.7%)	1362	70.6	859	44.6	63.1
5	2265	2125 (93.8%)	954	44.9	462	21.7	48.4
10	2318	2114 (91.2%)	410	19.4	84	4.0	20.5
15	2015	1814 (90.0%)	395	21.8	28	1.5	7.1
20	2072	1881 (90.8%)	786	41.8	13	0.7	1.7
30	2081	1959 (94.1%)	1230	62.8	1	0.1	0.1
40	2020	1902 (94.2%)	1535	80.7	0	0	0
60	1935	1840 (95.1%)	1679	91.3	0	0	0
80	716	645 (90.1%)	536	83.1	0	0	0
100	823	723 (87.8%)	665	92.0	0	0	0

A total of 227 normal tadpoles produced from five matings (Nos. 1~5) were examined in terms of chromosome number and nuclear size by making use of their tail-tips. It was found that all of them were diploids, although some tad-

with UV-irradiated sperm

No. of tail-bud embryos		No. of embryos at the hatching stage		No. of 40-day-old tadpoles	No. of metamorphosed frogs
Normal	Abnormal	Normal	Abnormal		
1472 (96.1%)	31 (2.0%)	1455 (95.0%)	17 (1.0%)	1421 (92.8%)	1368 (89.3%)
1362 (70.6%)	357 (18.5%)	859 (44.6%)	503 (26.1%)	695 (36.0%)	593 (30.8%)
954 (44.9%)	651 (30.6%)	462 (21.7%)	492 (23.2%)	348 (16.4%)	270 (12.7%)
410 (19.4%)	823 (38.9%)	84 (4.0%)	326 (15.4%)	44 (2.1%)	30 (1.4%)
395 (21.8%)	686 (37.8%)	28 (1.5%)	367 (20.2%)	12 (0.7%)	8 (0.4%)
786 (41.8%)	663 (35.2%)	13 (0.7%)	773 (41.1%)	6 (0.3%)	0
1230 (62.8%)	383 (19.6%)	1 (0.05%)	1229 (62.7%)	11 (0.6%)	0
1535 (80.7%)	196 (10.3%)	0	1535 (80.7%)	20 (1.1%)	0
1679 (91.3%)	88 (4.8%)	0	1679 (91.3%)	7 (0.4%)	0
536 (83.1%)	47 (7.3%)	0	536 (83.1%)	0	0
665 (92.0%)	31 (4.3%)	0	665 (92.0%)	0	0

raised from eggs inseminated with UV-irradiated sperm

the hatching stage						
	Abnormally shaped					
Total no.	Haploid type			Non-haploid type		
	No.	Percentage to normally cleaved eggs	Percentage to hatching embryos	No.	Percentage to normally cleaved eggs	Percentage to hatching embryos
17	0	0	0	17	1.1	1.2
503	12	0.6	0.9	491	25.5	36.0
492	16	0.8	1.7	476	22.4	49.9
326	82	3.9	20.0	244	11.5	59.5
367	230	12.7	58.2	137	7.6	34.7
773	596	31.7	75.8	177	9.4	22.5
1229	1122	57.3	91.2	107	5.5	8.7
1535	1514	79.6	98.6	21	1.1	1.4
1679	1676	91.1	99.8	3	0.2	0.2
536	536	83.1	100.0	0	0	0
665	665	92.0	100.0	0	0	0

poles had a few abnormal mitoses in addition to normal diploid ones. These abnormal mitoses were hypo- or hyperdiploid or had a dicentric or ring chromosome.

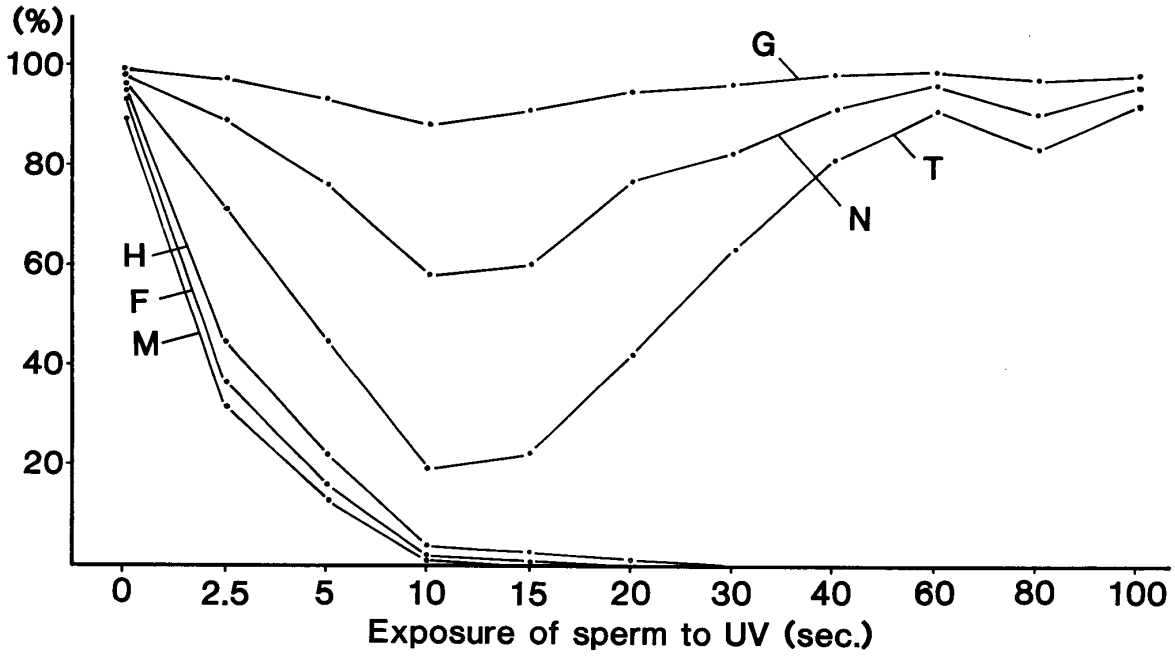

Fig. 1. Proportions of individuals at different developmental stages to normally cleaved eggs.

G: Gastrulae N: Neurulae T : Tail-bud embryos
H: Hatching embryos F : Feeding tadpoles M: Metamorphosed frogs

Of the 503 abnormal individuals at the hatching stage, 12 were of typical haploid-shape; their haploidy was confirmed by examining chromosomes and resting nuclei in their tail-tips and heads. The remaining 491 abnormal individuals which were not typical haploids in appearance were mostly hypodiploids having 21～25 chromosomes. Although there were diploid mitoses having 26 chromosomes, it was unknown whether each of these chromosomes was normal or not. In the metaphase spreads of the abnormal, non-haploid individuals, a dicentric or ring chromosome was frequently found (Tables 2, 3, 4; Fig. 4).

b. Irradiation for 5 seconds

A total of 2265 eggs obtained from 20 females were inseminated in 20 matings (Nos. 1～20) with sperm which had been obtained from 20 males and irradiated for 5 seconds. It was found that 2125 (93.8%) eggs in total, 76.6～100% of the respective number of eggs in each mating, cleaved normally. Of the normally cleaved eggs, 148, 372 and 651 died of various abnormalities at the gastrula, neurula and tail-bud stage, respectively. At the hatching stage, a total of 462 (21.7%) of the normally cleaved eggs, 5.5～67.7% in each mating, were normal, while 492 became abnormal. After this stage, 192 of the normal, hatched tadpoles died of ill-development, edema or some other abnormalities. Eventually, the remaining 270 (12.7%) tadpoles, 3.1～28.0% of the normally cleaved eggs in each mating, metamorphosed normally (Table 1; Fig. 1).

A total of 55 normal tadpoles produced from five matings (Nos. 1～5) were examined in terms of chromosome number and nuclear size. The results showed that each of them was a diploid, as almost all the metaphase spreads contained

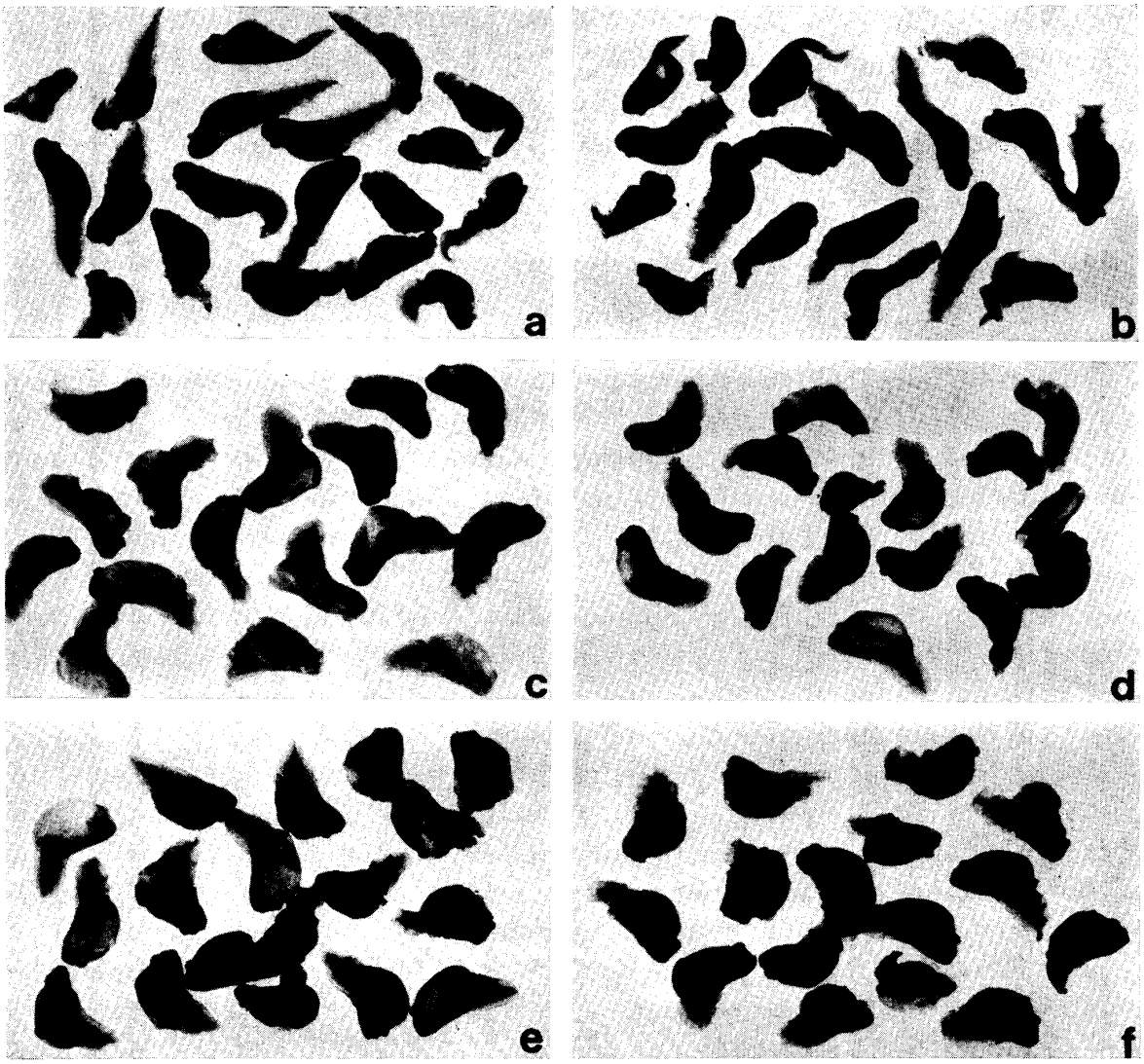

Fig. 2. Abnormal embryos at the hatching stage raised from eggs inseminated with irradiated sperm. ×2.5

a. Irradiation for 2.5 seconds
b. Irradiation for 5 seconds
c. Irradiation for 10 seconds
d. Irradiation for 15 seconds
e. Irradiation for 20 seconds
f. Irradiation for 30 seconds

26 chromosomes, although a few hypo- or hyperdiploid mitoses were found among them. Of the abnormal individuals, 16 were typical haploids in appearance. Their haploidy was confirmed by examining chromosome number and nuclear size in their tail-tips and heads.

The chromosomes and resting nuclei of the remaining 476 abnormal tadpoles were also examined in their tail-tips and heads (Fig. 2b). It was found that these abnormal individuals were mostly hypodiploids, whose mitoses contained 20~25 chromosomes. In the abnormal individuals other than haploid-shaped ones, there were no true haploids, although 13 individuals were mosaics including haploid cells (Tables 2, 3, 4; Fig. 4).

c. Irradiation for 10 seconds

A total of 2318 eggs obtained from 20 females were inseminated in 20 matings (Nos. 1~20) with sperm which had been obtained from 20 males and irradiated for 10 seconds. The results showed that 2114 (91.2%) eggs in total, 57.2~100% of the respective number of eggs in each mating, cleaved normally. Of the normally cleaved eggs, 256, 625 and 823 died of various abnormalities at the gastrula, neurula and tail-bud stage, respectively, while 84 and 326 were normal and abnormal at the hatching stage, respectively. Normal, hatched tadpoles were produced from 11 matings (Nos. 1, 3, 6, 7, 11~15, 19 and 20) and corresponded to 1.7~33.3% of the normally cleaved eggs in each mating, while no normal tadpoles were produced from the remaining nine matings.

Of the normal tadpoles at the hatching stage, 44 became feeding tadpoles and afterwards 30 produced from six matings (Nos. 3, 6, 11, 15, 19 and 20) metamorphosed normally, while the other 14 died of various abnormalities before metamorphosis (Table 1; Fig. 1). Of the 326 abnormal individuals at the hatching stage, 82 were of typical haploid-shape. Two of them which had been produced from mating No. 19 were living as feeding tadpoles at the age of 40 days, although they could not attain the metamorphosing stage. All the other abnormal individuals at the hatching stage died before eating.

The 84 normal tadpoles at the hatching stage were diploids when chromosomes and resting nuclei were examined in their tail-tips. In contrast, the haploidy of the 82 individuals which were typical haploids in appearance was confirmed by examining chromosomes and resting nuclei in their tail-tips. The remaining 244 abnormal individuals were mostly aneuploids whose mitoses contained 14~24 chromosomes. In these abnormal individuals other than haploid-shaped ones, there were no true haploids, although 41 of them were mosaics including haploid cells (Tables 2, 3, 4; Figs. 2, 4).

d. Irradiation for 15 seconds

A total of 2015 eggs obtained from 20 females were inseminated in 20 matings (Nos. 1~20) with sperm which had been obtained from 20 males and irradiated for 15 seconds. It was found that 1814 eggs (90.0%) in total, 48.9~98.5% of the respective number of eggs in each mating, cleaved normally. Of the normally cleaved eggs, 165, 568 and 686 died of various abnormalities at the gastrula, neurula and tail-bud stage, respectively. At the hatching stage, only 28 were normal tadpoles, while 367 were abnormal. Of the normal tadpoles, 13 were produced from mating No. 6, while 15 were from mating No. 11. These two numbers of tadpoles corresponded to 19.7% and 16.1% of the respective number of normally cleaved eggs. From the other 18 matings, no normal tadpoles were produced. Six tadpoles derived from mating No. 6 and two from mating No. 11 metamorphosed normally, while the other 20 tadpoles died of various abnormalities before metamorphosis (Table 1; Fig. 1).

The 28 normally shaped tadpoles at the hatching stage were diploids; there were no haploids among them when their chromosomes and resting nuclei were

examined in their tail-tips. Of the 367 abnormal individuals at the same stage, 230 were of typical haploid-shape in appearance. It was verified that all of them were haploids in fact by counting the chromosome number and measuring the nuclear size in their tail-tips and heads. Among the remaining 137 abnormal individuals there were no true haploids. They were mostly aneuploids, although 36 of them were mosaics including haploid cells (Tables 2, 3, 4; Figs. 2, 4).

e. Irradiation for 20 seconds

A total of 2072 eggs obtained from 20 females were inseminated in 20 matings (Nos. 1～20) with sperm which had been obtained from 20 males and irradiated for 20 seconds. The results showed that 1881 (90.8%) eggs in total, 41.9～99.4% of the respective number of eggs in each mating, cleaved normally. Thereafter, 104, 328 and 663 of the normally cleaved eggs died of various abnormalities at the gastrula, neurula and tail-bud stage, respectively. At the hatching stage, there were 13 normal and 773 abnormal individuals. The normal individuals included 12 produced from mating No. 6 and one from mating No. 11. No normal individuals were produced from the other 18 matings. After the hatching stage, 10 of the normal individuals died of edema or ill-development, while the other three became feeding tadpoles. The latter were underdeveloped and died before metamorphosis, although they were living at the age of 40 days. Of the abnormal individuals at the hatching stage, 596 were of typical haploid-shape (Fig. 3b). Of these haploid-shaped individuals, three produced from matings Nos. 6 and 19 became feeding tadpoles and were living at the age of 40 days, although they could not attain the metamorphosing stage. All the abnormal individuals other than these three died shortly after hatching.

The examination of chromosome number and nuclear size showed that the 13 normally shaped individuals at the hatching stage were diploids. Of the 596 individuals of typical haploid-shape, 258 produced from 10 matings (Nos. 1～10) were all haploids in fact, while there were no true haploids among the 177 abnormal individuals other than the haploid-shaped ones. They were mostly aneuploids, although 21 of them were mosaics including haploid cells (Tables 2,

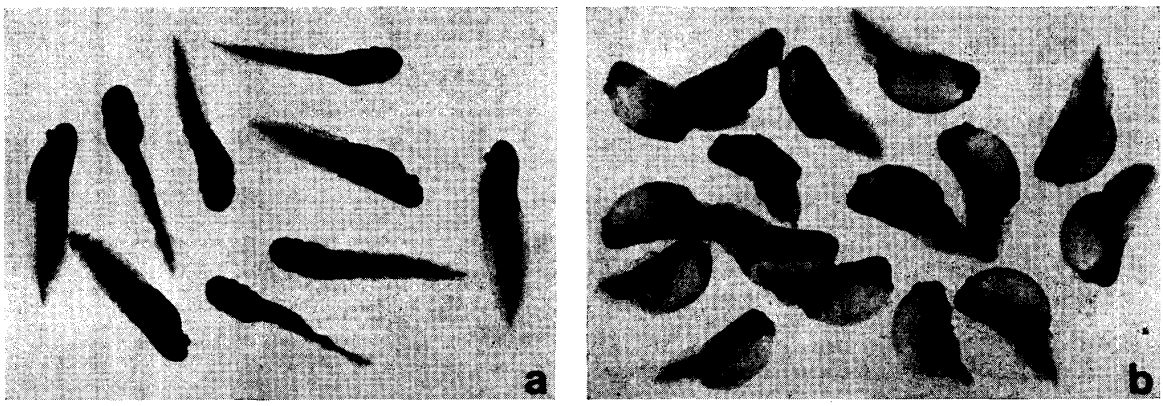

Fig. 3. Haploid embryos raised from eggs inseminated with sperm irradiated for 20 seconds. ×2.5
a. Control diploids b. Haploid embryos at the hatching stage

3, 4; Figs. 2, 4).

f. Irradiation for 30 seconds

A total of 2081 eggs obtained from 20 females were inseminated in 20 matings (Nos. 1~20) with sperm which had been obtained from 20 males and irradiated

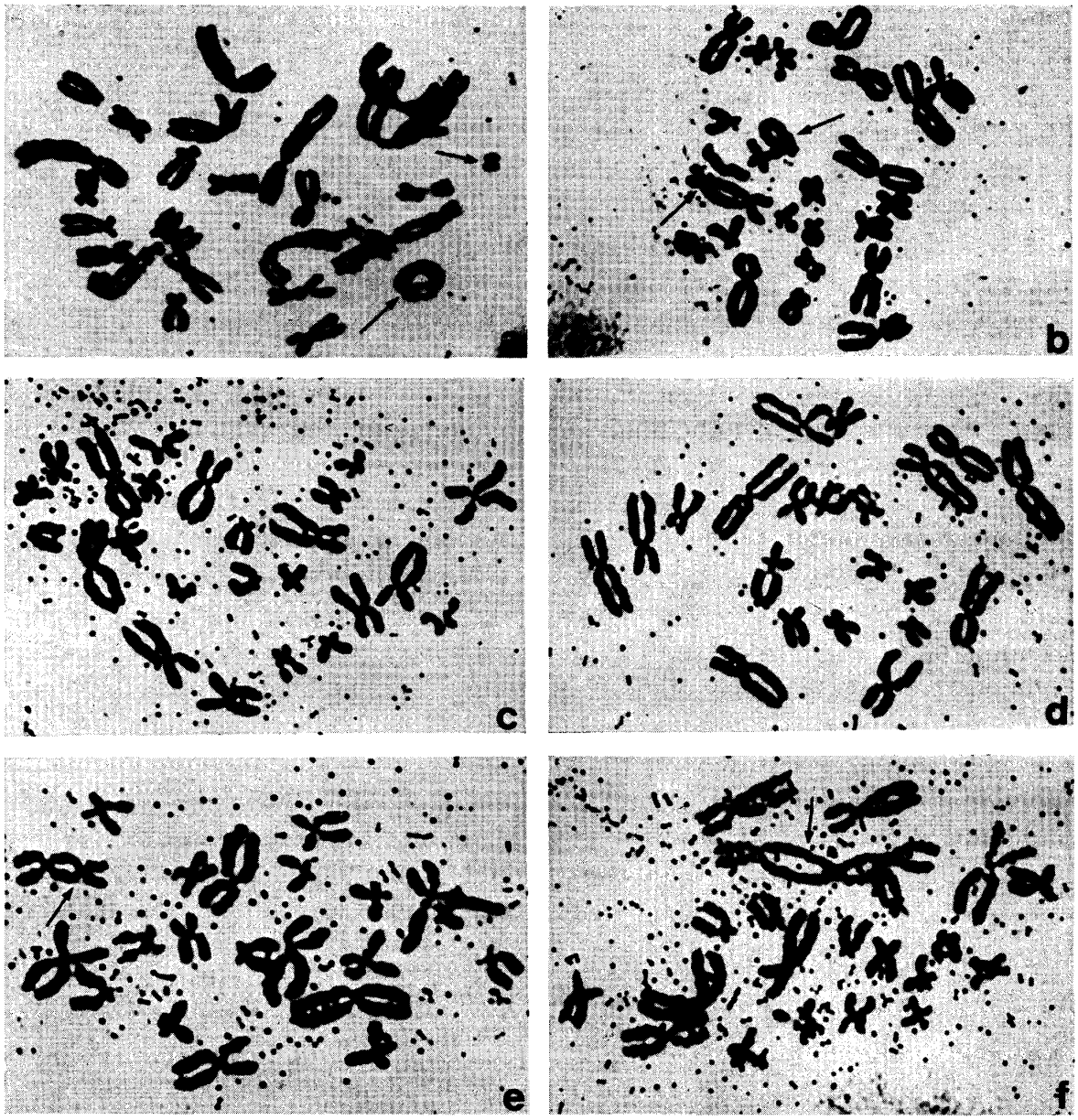

Fig. 4. Chromosome aberrations in abnormal embryos at the hatching stage raised from eggs inseminated with irradiated sperm. ×1500

a. Irradiation for 2.5 seconds, 27 chromosomes including a ring and a fragment
b. Irradiation for 5 seconds, 25 chromosomes including a dicentric chromosome and a deficient chromosome
c. Irradiation for 5 seconds, 24 chromosomes
d. Irradiation for 10 seconds, 22 chromosomes
e. Irradiation for 20 seconds, 21 chromosomes including a dicentric chromosome
f. Irradiation for 20 seconds, 21 chromosomes including a tricentric chromosome

for 30 seconds. It was found that 1959 (94.1%) eggs in total, 86.2~100% of the respective number of eggs in each mating, cleaved normally. Thereafter, 88, 258 and 383 eggs died of various abnormalities at the gastrula, neurula and tail-bud stage, respectively. At the hatching stage, only one individual produced from mating No. 6 was normal, while 1229 were abnormal. This normal individual died of edema at the feeding tadpole stage. Of the abnormal individuals at the hatching stage, 1122 were of typical haploid-shape. Of the latter,

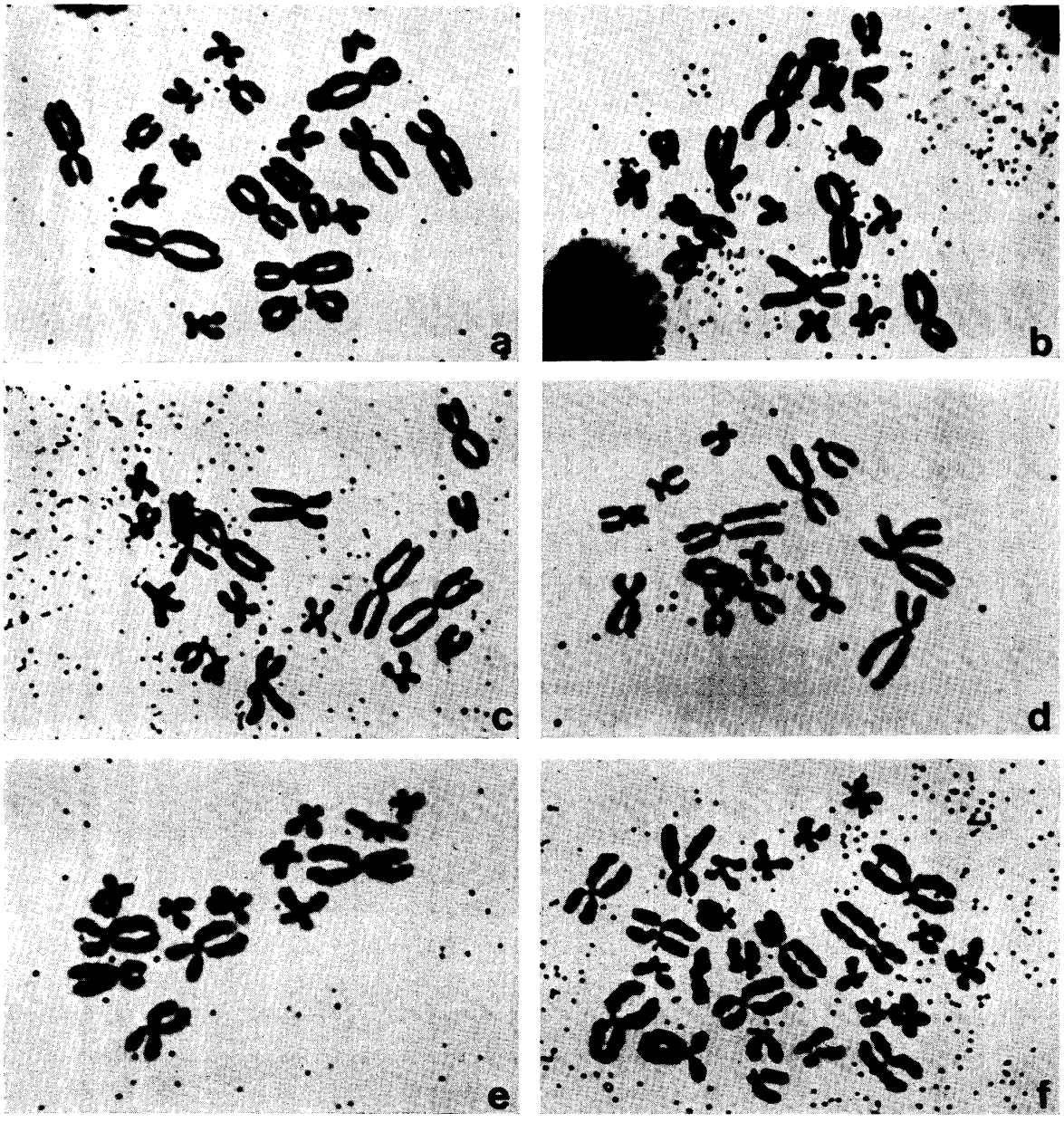

Fig. 5. Chromosome aberrations in abnormal embryos at the hatching stage raised from eggs inseminated with irradiated sperm. ×1500

a. Irradiation for 30 seconds, 20 chromosomes
b. Irradiation for 40 seconds, 18 chromosomes
c. Irradiation for 30 seconds, 17 chromosomes
d. Irradiation for 40 seconds, 14 chromosomes
e. Irradiation for 40 seconds, 13 chromosomes
f. Control, 26 chromosomes

11 produced from matings Nos. 6, 18 and 19 became feeding tadpoles and were living at the age of 40 days. However, they were underdeveloped and died before metamorphosis.

Of the typically haploid-shaped individuals, 484 produced from 10 matings (Nos. 1～10) were confirmed to be true haploids by examining chromosomes and resting nuclei in their tail-tips and heads. Among the 107 abnormal individuals other than the haploid-shaped ones, there were no true haploids when chromosome number and nuclear size were examined. They were mostly aneuploids, although five were mosaics including haploid cells (Tables 2, 3, 4; Figs. 2, 5).

g. Irradiation for 40 seconds

A total of 2020 eggs obtained from 20 females were inseminated in 20 matings (Nos. 1～20) with sperm which had been obtained from 20 males and irradiated for 40 seconds. It was found that 1902 (94.2%) eggs in total, 83.3～99.3% of the respective number of eggs in each mating, cleaved normally. Thereafter, 45, 126 and 196 died of various abnormalities at the gastrula, neurula and tail-bud stage, respectively. At the hatching stage, there were 1535 abnormal individuals and no normal ones. Of these abnormal individuals, 1514 in total were of typical haploid-shape. Twenty of them including six from mating No. 6, seven from mating No. 18 and seven from mating No. 19, became feeding tadpoles and were living at the age of 40 days, although they could not attain the metamorphosing stage.

Of the typically haploid-shaped individuals, 324 produced from five matings (Nos. 1～5) were confirmed to be true haploids by counting the chromosome number and measuring the nuclear size. No true haploids were found in the 21 abnormal individuals other than haploid-shaped ones. They were mostly aneuploids and mosaics (Tables 2, 3, 4; Figs. 2, 5).

h. Irradiation for 60 seconds

A total of 1935 eggs obtained from 20 females were inseminated in 20 matings (Nos. 1～20) with sperm which had been obtained from 20 males and irradiated for 60 seconds. The results showed that 1840 (95.1%) eggs in total, 88.3～97.6% of the respective number of eggs in each mating, cleaved normally. Thereafter, 19, 54 and 88 of the normally cleaved eggs died of various abnormalities at the gastrula, neurula and tail-bud stage, respectively. At the hatching stage, 1679 individuals were living and all of them were abnormal. Of these individuals, 1676 were of typical haploid-shape. Only seven haploids produced from matings Nos. 6 and 18 became feeding tadpoles and were living at the age of 40 days, while all the other abnormal individuals died without eating. None of the feeding haploid tadpoles could attain the metamorphosing stage.

Chromosome number and nuclear size were examined in the tail-tips and heads of 495 haploid-shaped individuals produced from five matings (Nos. 1～5). The results indicated that all of them were true haploids. It was found that the three abnormal individuals other than the typically haploid-shaped ones were

aneuploids (Tables 2, 3, 4; Figs. 2, 5).

i. Irradiation for 80 or 100 seconds

A total of 716 eggs obtained from 13 females (Nos. 1～13) were inseminated in 13 matings with sperm which had been obtained from 13 males (Nos. 1～13) and irradiated for 80 seconds. It was found that 645 (90.1%) eggs in total cleaved normally. Of the normally cleaved eggs, 536 (83.1%) were living at the hatching stage.

A total of 823 eggs obtained from the same 13 females as the above were inseminated with sperm which had been obtained from the same 13 males and irradiated for 100 seconds. The results showed that 723 (87.8%) eggs cleaved normally. Of these normally cleaved eggs, 665 (92.0%) were living at the hatching stage. All the living individuals raised from eggs irradiated for 80 or 100 seconds were of typical haploid-shape. The haploidy of these individuals was not confirmed by cytological observations.

2. Relationship between survivals and UV doses

The percentages of normally cleaved eggs in the 10 experimental series and the control were nearly equal to one another, as described above. They were 87.8～95.1% in the experimental series, while it was 94.5% in the control.

TABLE 3

Percentages of embryos at the hatching stage raised from eggs of 20 females by insemination with UV-irradiated sperm. Thick letter indicates the UV dose which induced the lowest viability of normally cleaved eggs

Mating no.	Dose of UV								
	0 sec. (Cont.)	2.5 sec.	5 sec.	10 sec.	15 sec.	20 sec.	30 sec.	40 sec.	60 sec.
1	95	82	27	**2**	17	30	69	72	85
2	100	68	**25**	33	53	62	70	83	84
3	94	75	62	**15**	18	53	72	88	96
4	96	66	19	**4**	5	33	68	85	87
5	94	60	29	**7**	9	41	53	79	95
6	99	88	77	60	**21**	51	57	90	95
7	92	74	62	20	**10**	43	69	87	98
8	100	74	36	21	**12**	53	77	81	96
9	94	56	33	**10**	30	34	47	66	92
10	96	66	64	**11**	25	47	59	84	88
11	100	76	69	43	**18**	47	73	79	91
12	100	73	40	**13**	32	49	60	72	86
13	94	67	56	20	**11**	46	68	79	96
14	100	64	61	**30**	39	59	74	87	88
15	90	74	68	48	16	**0**	63	95	97
16	98	83	32	**1**	27	43	50	64	89
17	97	59	12	**8**	9	46	64	79	86
18	99	68	39	**5**	32	39	59	89	94
19	92	69	28	**12**	20	44	55	70	94
20	97	68	53	**23**	25	34	60	79	92
Mean	96	71	45	**19**	22	42	63	81	91

The percentages of survivals including normal and abnormal individuals at the hatching stage to normally cleaved eggs in 20 matings of each series are presented in Table 3. In mating No. 2, 5-second irradiation of sperm resulted in the lowest percentage among those of survivals produced by the eight kinds of doses. To be precise, 25% of the normally cleaved eggs derived from spermatozoa irradiated for 5 seconds were living at the hatching stage, while the percentages of survivals derived from spermatozoa irradiated for shorter or longer periods than 5 seconds were significantly higher than 25%. Ten-second irradiation of spermatozoa resulted in the lowest percentage among those of survivals produced by the eight kinds of doses in each of 13 mating groups, that is, 2%, 15%, 4%, 7%, 10%, 11%, 13%, 30%, 1%, 8%, 5%, 12%, and 23% of the respective number of normally cleaved eggs in matings Nos. 1, 3, 4, 5, 9, 10, 12, 14, 16, 17, 18, 19 and 20, respectively. Fifteen-second irradiation of sperm resulted in the lowest percentage of survivals in each of five mating groups, that is, 21%, 10%, 12%, 18% and 11% of the respective number of normally cleaved eggs in matings Nos. 6, 7, 8, 11 and 13, respectively. Twenty-second irradiation of spermatozoa resulted in no survivals in mating No. 15, while there were 16 or more percent of survivals when spermatozoa were irradiated for longer or shorter periods than 20 seconds in the same mating group.

When eggs were inseminated with sperm irradiated for 2.5 or 5 seconds, comparatively many normal, hatched tadpoles were produced in each of the 20 matings. When they were inseminated with sperm irradiated for 10 seconds, no normal tadpoles were obtained in nine (Nos. 2, 4, 5, 8, 9, 10, 16, 17 and 18) matings. When they were inseminated with sperm irradiated for 15 or 20 seconds, a small number of normal tadpoles were obtained in only two matings, Nos. 6 and 11, while the other 18 matings did not produce such tadpoles. When eggs were inseminated with sperm irradiated for 30 seconds, a single normal tadpole was produced in mating No. 6 only. In the case of insemination with sperm irradiated for 40 or more seconds, no normal tadpoles were produced.

When eggs were inseminated with sperm irradiated for 2.5 or 5 seconds, metamorphosed frogs were produced in each of the 20 matings. When they were inseminated with sperm irradiated for 10 seconds, metamorphosed frogs were obtained in six matings, Nos. 3, 6, 11, 15, 19 and 20 at the rates of 6.3%, 13.7%, 5.2%, 4.0%, 1.8% and 2.5% of the respective number of normally cleaved eggs, respectively, while no metamorphosed frogs were produced in the other 14 matings. When eggs were inseminated with sperm irradiated for 15 seconds, metamorphosed frogs were produced in only two of the 20 matings. To be precise, 9.1% and 2.2% of the respective number of normally cleaved eggs became normally metamorphosed frogs in matings Nos. 6 and 11, respectively. In the case of irradiation of spermatozoa for 20 or more seconds, no metamorphosed frogs were produced.

3. Relationship between haploids and UV doses

The eggs inseminated with sperm irradiated for 10 seconds were the lowest in

percentage of survivals at the hatching stage to normally cleaved eggs among those inseminated with sperm irradiated for 2.5 to 60 seconds. Although the survivals somewhat varied in percentage from mating to mating, they were 19% on the average of 20 matings (Table 3). The percentages of haploids to survivals at the hatching stage in the 20 matings of each series are presented in Table 4. In the series of eggs inseminated with sperm irradiated for 10 seconds, 0~88%, average of 24%, of the survivals at the hatching stage in the 20 matings were haploids. The rate of haploids to survivals at the hatching stage increased rapidly as the period of irradiation became longer than 10 seconds, while it was extremely low when the period of irradiation was 2.5 or 5 seconds. Although the 20 matings of each series somewhat differed from one another in the rate of haploids to survivals as well as in the rate of survivals to normally cleaved eggs, the lowest mating in producing survivals in the eight experimental series of the same mating number was usually the forerunner of the mating in which more than 70% of survivals were haploids (Tables 3, 4). In mating No. 2, for example, 88% of the survivals were haploids when sperm were irradiated for 10 seconds, while only 8% were haploids when they were irradiated for 5 seconds. In mating No. 3, 75% of the survivals were haploids in the series of 15-second irradiation, while only 8% were haploids in the series of 10-second irradiation. In mating No. 6, 75% of the survivals were haploids in the series of 20-second

TABLE 4

Percentages of typical haploid embryos at the hatching stage raised from eggs of 20 females by insemination with UV-irradiated sperm. Thick letter indicates the UV dose which induced the lowest viability of normally cleaved eggs

Mating no.	Dose of UV								
	0 sec. (Cont.)	2.5 sec.	5 sec.	10 sec.	15 sec.	20 sec.	30 sec.	40 sec.	60 sec.
1	0	1	5	**50**	80	88	93	98	99
2	0	3	**8**	88	77	88	95	100	100
3	0	2	3	**8**	75	82	93	99	100
4	0	2	6	**20**	40	71	92	99	100
5	0	0	0	**50**	40	71	94	100	100
6	0	0	0	7	**7**	75	93	100	100
7	0	0	0	17	**22**	72	88	99	100
8	0	0	0	17	**33**	70	91	100	99
9	0	0	0	**27**	71	79	95	100	100
10	0	0	0	**25**	83	80	93	99	100
11	0	0	0	5	**19**	73	82	99	100
12	0	3	6	**27**	72	77	90	100	100
13	0	0	0	4	**42**	71	88	98	100
14	0	3	6	**26**	75	76	91	98	100
15	0	1	2	10	35	**0**	96	98	99
16	0	1	3	**0**	74	78	97	99	100
17	0	0	0	**24**	50	73	84	98	100
18	0	2	4	**33**	76	80	95	98	100
19	0	1	2	**23**	74	78	93	98	100
20	0	1	1	**14**	55	71	90	99	100
Mean	0	1	2	**24**	55	73	92	99	100

irradiation, while 7% were haploids in the series of 15-second irradiation. A similar situation was also found in 13 other matings. In the remaining four (Nos. 4, 5, 17 and 20) matings, 71%, 71%, 73% and 71% of the respective number of survivals were haploids in the series of 20-second irradiation, while 20%, 50%, 24% and 14% in the series of 10-second irradiation and 40%, 40%, 50% and 55% in the series of 15-second irradiation were haploids.

In the series of 30-second irradiation, 82~97%, average of 92%, of the respective number of survivals at the hatching stage were haploids, while 98~100%, average of 99%, and 99~100% were haploids in the series of 40-second irradiation and 60-second irradiation, respectively. When eggs had been inseminated with sperm irradiated for 80 or 100 seconds, all the survivals at the hatching stage were haploids.

4. Viability and external characters of haploids

A small number of haploids became feeding tadpoles and lived for more than 40 days, while the others died without eating. All the feeding haploid tadpoles were raised from the eggs of females Nos. 6, 18 and 19. While 257 haploids were produced from eggs of female No. 6 by insemination with sperm irradiated for 10~60 seconds, 12 (4.7%) of them became feeding tadpoles. Of these haploids, 1, 2, 6 and 3 were produced by sperm irradiated for 20, 30, 40 and 60 seconds, respectively. Of 343 haploids raised from eggs of female No. 18 by insemination with sperm irradiated for 2.5~60 seconds, 14 (4.1%) became feeding tadpoles. Three, 7 and 4 of these feeding tadpoles were produced by sperm irradiated for 30, 40 and 60 seconds, respectively. Of 354 haploids raised from eggs of female No. 19 by insemination with sperm irradiated for 2.5~60 seconds, 17 (4.8%) became feeding tadpoles. Of the latter, 2, 2, 6 and 7 were produced by sperm irradiated for 10, 20, 30 and 40 seconds, respectively. Of all the feeding tadpoles stated above, none could attain the metamorphosing stage.

The most typical shape of haploids at the hatching stage is shown in Fig. 3b. They were microcephalic and edematous and had a tail which was short and vertically wide. These haploids usually died of severe ascites without taking food. In contrast to these inviable haploids, the viable ones which became feeding tadpoles were scarcely edematous at the hatching stage, although their tails were distinctly shorter than those of normal diploids. In the series of 2.5- and 5-second irradiation, 503 (36.9%) of the 1362 and 492 (51.6%) of the 954 survivals, respectively, raised from eggs of 20 females (Nos. 1~20) were scarcely of typical haploid-shape; while their heads resembled those of normal diploid and their tails were long and vertically narrow (Fig. 2a, b). The individuals with such an abnormal shape were usually hypodiploids. In the series of 10-, 15- and 20-second irradiation, 326 (79.5%) of the 410, 367 (92.9%) of the 395 and 773 (98.3%) of the 786 survivals, respectively, at the hatching stage were abnormal (Fig. 2c, d, e). In these series, survivals having a typical haploid shape gradually increased from 20.0% to 75.8% with increase of dosage. In the series of 30-, 40- and 60-second irradiation, 99.9% or 100% of survivals were

abnormal and 91.2~99.8% were of typical haploid-shape. The abnormal individuals other than the haploid-shaped ones were confirmed to be mostly aneuploids situated in chromosome constitution between haploid and diploid by examining the chromosome number in the tail-tips and heads (Fig. 2f).

DISCUSSION

BARDEEN (1907) was probably the earliest investigator who produced amphibian haploids by irradiated sperm. The toad tadpoles which he obtained from eggs inseminated with X-irradiated sperm showed deformities characteristic of haploids, although he did not refer to them as haploidy. Voluminous studies on the effects of gametes exposed to radium bromide or mesothorium in amphibian development were carried out by HERTWIG's family. The so-called HERTWIG effect was discovered in *Rana fusca* (=*temporaria*) by O. HERTWIG (1911) and G. HERTWIG (1911); they irradiated sperm and unfertilized eggs for various periods of time and observed that eggs derived from irradiated gametes developed more abnormally if these gametes were irradiated for a short time than if they were irradiated for a long time. The occurrence of gynogenetic haploids by insemination with irradiated sperm was confirmed in *Rana fusca* by P. HERTWIG (1913) and in *Triton* by O. HERTWIG (1913), while that of androgenetic haploids by insemination of irradiated unfertilized eggs with normal sperm was observed in *Triton* by P. HERTWIG (1916). G. HERTWIG (1913) reported that gynogenetic haploids were produced in *Bufo vulgaris* (=*bufo*) or *Rana esculenta* by inseminating their eggs with irradiated foreign sperm.

SIMON (1930) exposed sperm or unfertilized eggs of *Rana fusca* to radium, X-rays or UV-rays before insemination and confirmed the HERTWIG effect as a whole. DALCQ (1930) made a cytological interpretation to the HERTWIG effect by observing the behavior of irradiated chromosomes in developing eggs. According to SIMON and DALCQ, UV-rays were the most effective among the three kinds of radial agents in producing haploid larvae by irradiation of spermatozoa, although SIMON found that UV-rays were ineffective on the eggs of *Rana fusca* owing to the pigmentation of the animal pole. SELMAN (1958) utilized UV-rays in producing gynogenetic haploids in European newts. He obtained a high proportion of haploids by inseminating eggs with UV-irradiated sperm in three *Triturus* species.

RUGH (1939) and HENSHAW (1943) confirmed the HERTWIG effect in *Rana pipiens* by irradiating sperm with X-ray doses ranging from 15 r to 50,000 r. According to RUGH, the hatching percentage gradually decreased as the X-ray doses were increased and became 1.6% at 10,000 r, while it began to increase as the doses were furthermore increased and reached 90.5% at 50,000 r. The embryos derived from sperm exposed to the largest dose of X-rays were of morphologically uniform haploid-shape. RUGH and EXNER (1940) obtained a high percentage of gynogenetic haploids from *Rana pipiens* eggs by inseminating with *Rana catesbeiana* sperm irradiated with X-rays at 66,000 r. BRIGGS, GREEN and

KING (1951) also inseminated *Rana pipiens* eggs with irradiated foreign sperm. When eggs were inseminated with heavily X-irradiated (65,300 r or 98,000 r) *Rana catesbeiana* or *pipiens* sperm, they developed as typical gynogenetic haploids.

The present study was planned in 1966 to establish the best method to produce gynogenetic haploids by UV-irradiated sperm in the Japanese brown frog, *Rana japonica,* and was carried out in the following year in parallel with the study on production of gynogenetic haploids by toluidine blue treatment of sperm (NISHIOKA and KONDO, 1978). The proportions of gastrulae, neurulae, tail-bud embryos and hatched tadpoles to normally cleaved eggs derived from sperm irradiated with UV-rays for incremental periods of time ranging from 2.5 to 60 seconds were very similar to those derived from sperm treated with toluidine blue solution for periods ranging from 5 to 60 minutes (Fig. 1, cf. NISHIOKA and KONDO, 1978, Fig. 1). The proportions of total individuals, normal diploids and typical haploids at the hatching stage to normally cleaved eggs derived from UV-irradiated sperm were also very similar to those derived from toluidine blue-treated sperm (Fig. 6, cf. NISHIOKA and KONDO, 1978, Fig. 4). These findings seem to indicate that UV-rays have the same action resulting in inactivation of sperm chromosomes as toluidine blue has. At the hatching stage 98% and 100% of the survivals derived from sperm exposed to toluidine blue solution for 40 and 60 minutes, respectively, were haploids and 99% and 100% of the survivals derived from sperm exposed to UV-rays for 40 and 60 seconds, respectively, were also haploids.

POGANY (1971, 1973) reported in *Rana pipiens* that low-dose embryos were most severely affected in spite of their diploid condition, while high-dose embryos were all haploids. Thereafter, POGANY (1976) exposed spermatozoa to

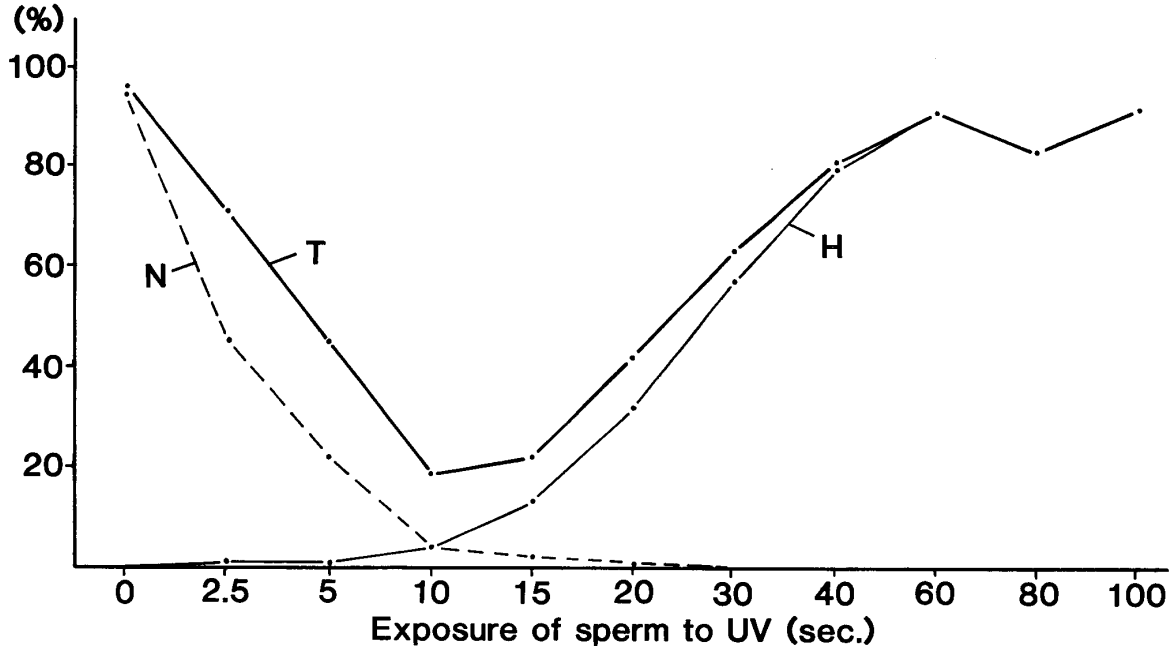

Fig. 6. Proportions of total, normally shaped and haploid type embryos at the hatching stage to normally cleaved eggs.

H: Haploid type embryos N: Normally shaped embryos T: Total embryos

UV-rays for incremental periods of time ranging from 5 to 30 seconds and found that the initial decrease in survival at lower doses (up to 8 sec) was not accompanied by any chromosomal losses. Eight second cultures were almost exclusively composed of abnormal embryos which were concomitant with aneuploid chromosomal conditions, while haploidy was obtained very abruptly at 9 second exposure. In the present study, the proportions of normally diploid-shaped, typically haploid-shaped and the other abnormal embryos to the total number of embryos were very similar to those reported by NISHIOKA and KONDO in the study of insemination with toluidine blue-treated sperm (Fig. 7, cf. NISHIOKA and KONDO, 1978, Fig. 5). In these two studies, the proportions of abnormal, non-haploid-type embryos showed a comparatively low normal curve between zero and 60 second exposure to UV-rays or between zero and 60 minute exposure to toluidine blue, in contrast to those reported by POGANY (1976).

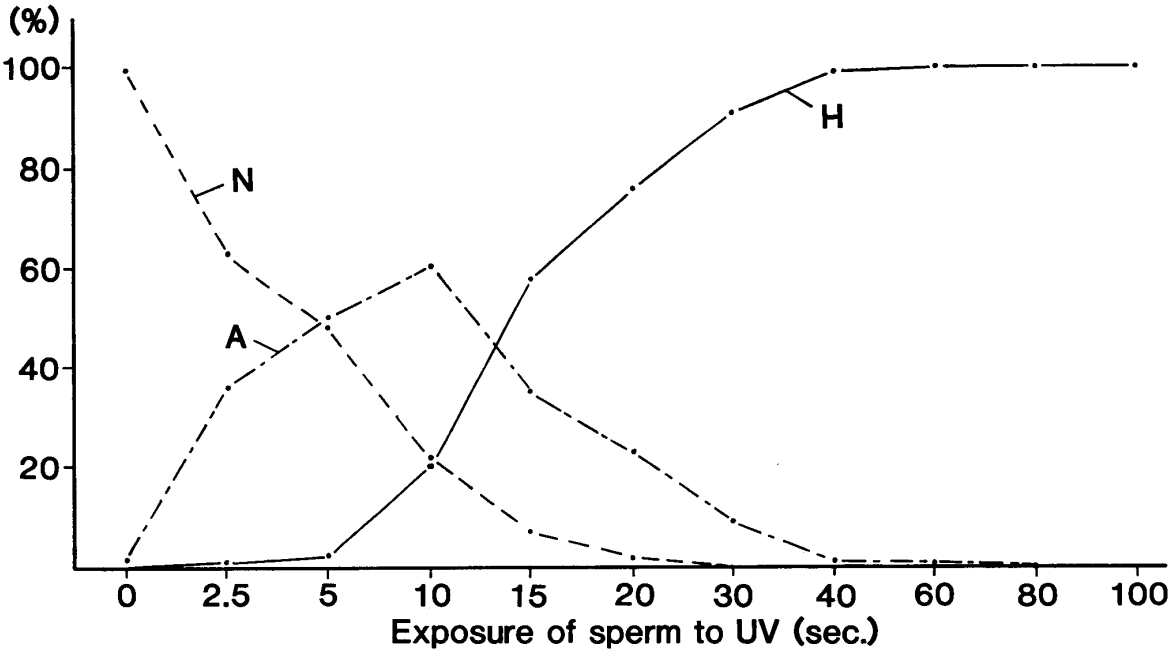

Fig. 7. Proportions of normally shaped, haploid type and non-haploid abnormal type embryos to total ones at the hatching stage.

A: Non-haploid abnormal type embryos H: Haploid type embryos
N: Normally shaped embryos

From the three curves in Fig. 7 showing the proportions of normally diploid-shaped, typically haploid-shaped and the other abnormal, non-haploid-type embryos, it was very probable that a small UV dose damaged partly or completely the multiplying function of a few chromosomes of some spermatozoa, while a large dose completely destroyed the function of all the haploid chromosomes, as assumed by many investigators. The findings that there were a few abnormal mitoses being hyper- or hypodiploid or having a dicentric or ring chromosome in addition to normally diploid mitoses in some normally shaped tadpoles derived from sperm irradiated for 2.5 or 5 seconds, that abnormal, non-

haploid-type individuals derived from sperm irradiated for 2.5 or 5 seconds were mostly hypodiploids having 20~25 chromosomes, and that those derived from sperm irradiated for 10 or more seconds were mostly aneuploids having 14~25 chromosomes seemed to indicate that the above general assumption is correct. More detailed observations of chromosomes in viable and inviable individuals derived from sperm irradiated for a short time will further confirm the assumption that a small UV dose damages partly or completely the multiplying function of a few chromosomes and produces hypodiploids by inseminating eggs with such sperm. The results of experiments made on this line will be reported in Part II of this study.

SUMMARY

1. In order to establish the best method for producing gynogenetic haploids by making use of UV, eggs of the Japanese brown frog, *Rana japonica,* were inseminated with sperm irradiated at 24 erg/mm^2/sec for 2.5 to 100 seconds.

2. The proportions of normally cleaved eggs in the experimental and control series were nearly equal to each other. UV-irradiation of sperm evidently showed the HERTWIG effect in the development of inseminated eggs. The proportion of survivals including both normal and abnormal individuals at the hatching stage was usually the lowest when eggs were inseminated with sperm irradiated for 10 seconds. The longer or shorter than 10 seconds the period of irradiation, the higher was the rate of survivals.

3. When the period of irradiation was increased, normal diploids rapidly decreased in number, while gynogenetic haploids gradually increased. When eggs were inseminated with sperm irradiated for 30, 40 and 60 seconds, 57%, 80% and 91% of the normally cleaved eggs became typical haploids at the hatching stage, respectively. These haploids occupied 91%, 99% and 100% of the survivals, respectively. When eggs were inseminated with sperm irradiated for 80 or 100 seconds, all the survivals at the hatching stage were haploids.

4. A small number of haploids became feeding tadpoles, although they could not attain the metamorphosing stage. All of them were raised from eggs of three of 20 females by insemination with sperm irradiated for various periods. All the other haploids as well as the abnormal individuals other than haploids died without eating.

5. It was very probable that a small UV dose damaged partly or completely the multiplying function of a few chromosomes of some spermatozoa, while a large dose completely destroyed the function of all the haploid chromosomes.

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