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Title: EFFECTS OF FRACTIONATED RADIATION DOSES ON SURVIVAL TIMES OF THE NEWT (TARICHA GRANULOSA)

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Dr. David L. Willis

This was a study designed to determine the occurrence of recovery from radiation damage in Taricha granulosa. Irradiation with either gamma or x-ray sources in the range of 96-10,000 rads was given. (The mean or median survival times were determined.) Doses were fractionated and the survival times for the same total doses compared. The presence of a significant difference was interpreted as the existence of recovery.

Effects upon recovery by the factors of temperature, fraction interval, number of fractions, and dose rate were also tested. Temperature was of primary importance upon the occurrence and/or rate of recovery. If temperature was optimum for recovery the fraction interval based on the radiosensitivity of the cell cycle was the factor of next greatest importance. If the fractionation interval was too short, a merely additive effect of the fractions was observed and no recovery could be detected. The number of fractions seemed

to be an effective factor in allowing a significant difference in survival times only if the conditions of temperature and fraction interval were also met. Whether dose-rate has any effect upon recovery could not be determined conclusively from this study.

Effects of Fractionated Radiation Doses on Survival Times of the Newt (Taricha Granulosa)

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INTRODUCTION

Radiation biology as the study of the effects of ionizing radiation on biological systems came into being about the turn of this century. Comparing radiation effects between species has revealed a similarity of responses since as early as 1905, when Heineke studied pathological changes in mice, guinea pigs, and dogs. However, one of the most important points in comparative studies of radiosensitivity is that the criteria of damage must be the same.

Radiation damage was eventually traced to a cellular response. Actual definition of the mechanism of damage, however, was delayed until Puck and Marcus (1956) devised a mammalian cell culture technique. By plating cells in a single layer in culture and "harvesting" those in the same phase of the cell cycle, synchronized cell populations became available. Till and McCulloch (1961) felt that the mechanisms which caused cell death in such cell cultures probably were also active in causing lethality in whole organisms although a direct extrapolation could not be made. It was accepted thereafter that the same principles of damage mechanisms were at work in both *in vivo* and *in vitro* systems.

Irradiated mammals display characteristic response syndromes indicating the degree of damage to their various organ systems. "A

syndrome is defined as a collection of signs and symptoms characterizing the response of an organism to a particular stimulus, in this case, radiation" (Casarett, 1968). The time of appearance and duration of tissue changes are characteristic of an individual syndrome which is dependent upon the radiation dose the animal has received.

Four radiation syndromes are generally recognized. These are dose-related. At doses greater than 100,000 rads molecular death occurs. The organism dies during or immediately after administration of the radiation. Basic metabolic processes of the cells and tissues are presumably inactivated at this level. Above a dose of 10,000 rads, death follows in rodents within a day or two. This is termed the central nervous system (CNS) syndrome and, as the name suggests, it is caused by a breakdown of functions controlled by the central nervous system. Between 900-10,000 rads damage to the gastrointestinal (GI) system is manifested in loss of electrolytes and frequently diarrhea, which results in death in 3-5 days in rodents. Depending on the individual animal and the radiosensitivity of the species, death may or may not occur between 300 and 900 rads. Those that do die generally do so within 10-15 days and show changes in the blood and blood-forming organs. These are said to have died of the hematopoietic syndrome. Below 300 rads, in rodents, few animals die of the acute radiation syndrome which is the composite effect of several damaged systems causing death within a relatively short period of time after irradiation.

Within the framework of comparative radiation biology the similarities and differences of radiation responses between species is of

fundamental interest. Work has been done by Oliver (1964) on the dose response of plants (Vicia faba) as compared to mammals. Likewise, responses of poikilothermic animals such as amphibians and fish have been compared to mammals. All these various classes seem to have similarly shaped dose-response curves.

Amphibians, as a biologically important class in our environment, have been useful in radiation response studies because of their unique capacity for regeneration, and metamorphosis (Brunst, 1950). Modifications of these activities have acted as quantifiable measures of radiation damage. Amphibians, particularly urodeles (tailed-amphibians) have been shown to respond to irradiation in approximately the same dose range with basically the same syndromes as rodents (Allen et al., 1951; Conger and Clinton, 1973; Willis and Lappenbusch, 1976). According to Sparrow et al. (1970), urodeles, particularly Siredon mexicanum and Taricha granulosa showed all three characteristic radiation syndromes (CNS, GI, and hematopoietic) normally seen in rodents. For a comparative radiation response study with amphibians Taricha granulosa was the logical choice for my work, since it was so abundant locally. Previous work in this laboratory had shown it to be a hardy animal easily kept alive 10-12 months at 10°C without feeding.

Radiation response or survival studies have normally involved the administration of a range of radiation doses to a group of organisms, tabulation of deaths, and determination of the dose causing death of 50% of the organisms within 30 days. This dose was designated the $LD_{50/30}$ and used as a unit of comparative radiosensitivity between

species. However, when comparing the radiosensitivities of amphibians and rodents (on which the $LD_{50/30}$ idea was based) it has been found to be invalid. Amphibians have a long latent time before the occurrence of acute radiation effects are evident. Amphibians have not generally begun to visibly respond to radiation damage until after the observations on a rodent population would have already been terminated (i.e., 30 days).

In an attempt to demonstrate the inadequacies of comparing different vertebrate classes on a 30 day time span, A.H. Sparrow and associates (1970) compared the radiosensitivities of selected amphibians according to their nuclear and chromosomal values. If a 30 day interval was chosen to determine the LD_{50} it would have indicated that the larger the chromosomes in the nucleus of given amphibians the more radioresistant they were. However, when the LD_{50} was determined at later times post-irradiation it actually decreased with increasing nuclear and chromosomal volumes. Hence, the larger the nuclear and chromosomal volumes the more radiosensitive the organism. Taricha granulosa was among the species investigated. It was found to have relatively high nuclear and interphase chromosome volumes which led to a calculated LD_{50} of $\sim$ 170 rads.

Besides the innate radiosensitivity of the organism it has been demonstrated that the response of the organism can be affected by other factors. The most basic and important of these is temperature. This is especially important in poikilothermic organisms, such as amphibians. Work in this laboratory and elsewhere has explored the question

of what effect temperature played upon the latent time of the manifestation of acute radiation effects (Patt and Swift, 1948; Smith and Grenan, 1951; Willis and Prince, 1967). Results of the work in this laboratory with Taricha granulosa produced a survival time-dose curve for moderate and high doses of both gamma and x-ray sources at 10° and 20°C (Willis and Prince, 1967; Lappenbusch, 1969; Willis and Gruber, 1975; Willis and Lappenbusch, 1976). Additional data were needed at low doses to better define the LD_{50} for Taricha granulosa at 20°C.

In this laboratory and others it was found that the mean survival times in amphibians were inversely related to the temperature at which the animals were held post-irradiation (Patt and Swift, 1948; Smith and Grenan, 1951; Willis and Prince, 1967). As long as a low temperature was maintained after irradiation there seemed to be very little radiation damage manifested. If the temperature was returned to normal, however, appearance of acute radiation effects proceeded from that time at a normal rate (Allen et al., 1951; Berry and Oliver, 1964; Egami et al., 1967). A lower temperature seemed to greatly extend the latent period between irradiation and the appearance of acute radiation symptoms.

What actually takes place during the extended latent time at a lowered temperature has been the subject of considerable research. The question has been whether lowering the temperature inhibits the fixing of damage or whether it allows repair of the damage to occur (Winans et al., 1972). The terms "repair" and "recovery" are used interchangeably in the literature to mean to bring the system affected back to or as close to a pre-irradiation state as possible. The idea that recovery

from radiation damage occurred was discovered while trying to prove that the chromosomes were the sensitive sites of the cell (Elkind and Sutton, 1959).

Tests for recovery by giving a total dose in two or more fractions and comparing the survival time with that of a single dose soon became standard procedure. Hypo- or hyperthermia before, during, or after were often added variables. Experimentation for the occurrence of recovery has been extensive. Research with cell cultures has provided a description of the primary phases of recovery in relation to the initial dose and subsequent fractions (Elkind and Sutton, 1959, 1960; Elkind et al., 1965; Ben-Hur et al., 1974; Szechter and Schway, 1977). Rodents have generally been used for whole animal studies. Results have generally shown that fractionation does increase the amount of total dose needed to achieve the same cell-killing effectiveness as a single dose of radiation (Brown et al., 1960; Corp and Mole, 1966; Holloway et al., 1968).

Some Japanese workers are the only ones to have done extensive recovery studies with poikilothermic animals (Egami and Etoh, 1966; Egami et al., 1967; Etoh and Egami, 1967; Egami, 1969; Hyodo-Taguchi, 1970). They used fish, primarily Oryzias latipes. After initially determining a single-dose survival curve they added the variables of different numbers of fractions, different temperature, different fraction sizes, and different dose rates. They concluded that with fish temperature had the most critical effect upon acute radiation injury. The size of the initial dose could also affect the severity of the

response to the radiation. They felt, however, that the number of fractions and dose rate had no effect upon survival time.

Since the work of Sparrow et al. (1970) had suggested that amphibians reacted to radiation in much the same manner as mammals and the work of the Japanese with cold-blooded animals had suggested recovery took place according to the same patterns as in mammals, I felt an investigation of the question of recovery in Taricha granulosa was warranted. Although most of the basic survival time-dose curve had been completed for Taricha granulosa in this laboratory, no work using fractionated doses had been done. I first wanted to determine if recovery occurred in Taricha granulosa and if so, did it proceed according to expected patterns as determined in mammals and fish. If I was able to determine recovery, I then wanted to test whether it could be affected by different factors and whether these factors operated in a descending order of importance upon it. I hypothesized that I would find temperature most important; fractionation interval, second; number of fractions, third; and dose rate, fourth in importance on the effect they had upon recovery. Attempts to feed the newts would be made to determine whether survival time was severely affected by malnutrition.

MATERIALS AND METHODS

Collection and Maintenance of Newts

The newts used in this study were collected from four locations in western Oregon: The School of Agriculture experimental ponds off of Sulfur Springs Road ten miles north of Corvallis; Cronemiller Lake in the McDonald Experimental Forest near Peavy Arboretum; Hamer Lake two miles north of Nashville in the Coast Range; and the Gordon Lakes off Forest Service Road 1312 two miles east of Mountain House in the Cascades. The animals were transported back to the laboratory and either put in cold storage (10°C) or used for experiments directly. In either case all animals were allowed to acclimate to room temperature (20°C) for at least one week prior to irradiation. This temperature was maintained throughout all experiments.

It has been my observation that newts in captivity do not eat consistently. Efforts to feed all animals were made, but in three of the four experiments only approximately half of the newts could be observed eating with any regularity. However, in the fourth experiment in the Fall, 1977, all efforts to induce the animals to eat failed, so they were held unfed. Chopped liver, redworms, and earthworms were all tried as food. Redworms seemed to be preferred.

To facilitate the keeping of individual records on each animal, the newts were housed singly in small styrene boxes in the first two experiments. These small boxes were located in constant temperature water

baths. However, using the Cobalt-60 gamma source required irradiating ten animals in a single container at a time. This made it impossible to tell them apart throughout a series of fractionated irradiations. Thus, in the last two experiments, the newts were group housed in 20-30 gallon aquaria by dose.

The entire contents of each box and aquarium were changed once a week or oftener if they became excessively fouled. Water from three sources was used: natural well water from Oak Creek Fisheries Laboratory; artificial pond water (Alvarado, 1963); and distilled water. Chemical analyses of the well water and artificial pond water may be found in the Appendix (Worrest, 1975).

X-Irradiation

Newt irradiation was accomplished by use of a General Electric Maxitron 300 x-ray unit. The operating conditions were as follows: 300 kVp, 20 mA, 2 mm Cu internal filtration, no external filtration, and a target to source distance of 121 cm. A uniform field of exposure was achieved by use of a rotating lucite chamber inside which the newts were placed in individual, ventilated plastic vials. The chamber allowed 20 animals to be irradiated at a time. The dose-rate was checked with a Victoreen R-meter before each irradiation. Roentgen values were computed to equivalent rad values by the formula: 1 Roentgen = .96 rad in tissue.

Gamma-Irradiation

A Nuclear Systems Model R-60124 cobalt-60 source of 489 Ci strength was used for the gamma irradiation. The 25 cm diameter source chamber allowed ten newts in a 15 cm diameter plastic box to be irradiated simultaneously while unrestrained. The irradiation box contained approximately 60 ml of water to prevent dessication of the newts. If irradiation lasted longer than one hour, the chamber was aerated to supplement available oxygen. With this particular unit only two reliable dose rates were available: 28 rads/min and 1150 rads/min (Johnson, 1977).

Lappenbusch (1970) found that the radiosensitivity of Taricha granulosa fluctuated over a twenty-four hour period. Radiosensitivity appeared to be related to the light-dark cycle and circadian rhythm previously noted in these and other animals. The greatest change in survival time occurred when irradiation took place between nine p.m. and midnight. Therefore, all irradiations were completed before nine p.m. to avoid this complication.

Experimental Design

This investigation involved four experiments, the details of which are outlined in Table 1. In the literature the term "split" is commonly used to denote when a total dose is split into two doses of radiation and "fractions" to mean situations in which the dose is divided into

Table 1 Experimental outline of entire study specifying objectives and details of individual experiments

Water Source	Housing	Food	Type of Irradiation	Dose Rate (rads/min)	Doses Used (rads)	Number Newts Used		Purpose
EXPERIMENT 1 -- Collection date: February, 1976 -- Experiment duration: March 5, 1976 - October 28, 1976								
Natural Oak Creek water	Singly	Redworms	X-ray	41	Controls, 96, 192, 288, 384, 480	Controls: Experiment: Total:	50 189 239	1) determine survival time 2) note any gross morphological changes
EXPERIMENT 2 -- Collection date: August, 1976 -- Experiment duration: October 29, 1976 - January 18, 1977								
Artificial pond water	Singly	Redworms	X-ray	41	Controls, single dose or 2 fractions totaling 624	Controls: Experiment: Total:	120 132 252	1) test for evidence of recovery 2) test for any effect of temperature on recovery
EXPERIMENT 3 -- Collection date: February, 1977 -- Experiment duration: March 7, 1977 - June 17, 1977								
Artificial pond water	Group	Chopped liver	^{60}Co gamma	28	Controls, single dose or 2 or 4 fractions totaling 650, 2,000	Controls: Experiment: Total:	30 210 240	1) test for evidence of recovery 2) determine rate of recovery 3) determine any effect of fraction size on recovery
EXPERIMENT 4 -- Collection date: October, 1977 -- Experiment duration: October 18, 1977 - January 16, 1978								
Distilled water	Group	Unfed	^{60}Co gamma	28 and 1150	Controls, single dose or 2 or 4 fractions totaling 10,000	Controls: Experiment: Total:	168 202 370	1) determine dose rate effect on recovery 2) determine any effect of fractionating dose on recovery

any number of doses in excess of two. For the sake of simplicity, I have referred to any number of doses in excess of one as fractionated. Due to a calculation error at the time of exposure the four-fraction sequence of Experiment 3 was administered doses of ∿ 182 rads rather than 162 rads. All the experiments used death as an end point and survival time as the criterion of radiation response. Mean survival times were calculated for those groups having 100% mortality, taking into account unequal variances and, in some cases, unequal sample size. These were expressed as mean survival times (MST) ± either standard deviation (S.D.) or standard error (S.E.), whichever was most appropriate. Groups retaining survivors at the end of an observation period were represented by a smooth curve fit of the data points upon which a graphically determined median survival time was plotted (designated MST only). All experiments had groups of controls proportionate to the number of experimental animals. The limiting factor for the number of animals in each experimental and control group was the total number of newts available.

Post-Irradiation Procedures

Immediately after irradiation, the animals were returned to the laboratory and held at 20° ± 1°C for the duration of the experiment. Observations were made at least daily of all groups to determine mortalities. A lethal or threshold dose was one which was determined to

cause acute radiation death. Doses in excess of the threshold caused an increased percentage of mortality until 100% was reached.

During Experiment 1 weight at death was recorded to be compared to weight at irradiation and a simple autopsy performed to record any gross morphological changes. These observations were begun only after the 48th day because random autopsies prior to that time suggested some trends in organ damage relative to dose. The emphases of the damage seemed to shift with time, so Experiment 2 was designed with a sacrifice schedule in mind to allow these shifts to be followed more precisely.

To test for recovery in Experiment 2, a time interval had to be chosen which would maximize the effect of the dosage. Intestinal crypt cell cycle time is often used as an indicator of recovery. In this species the intestinal mucosa has a turnover time of 7.6 days at 23°-24°C (Filipy, 1977). Since cell activity is slower at lower temperatures, it was felt that allowing 10 days for completion of a cell cycle should be adequate at 20°C. Thus, 10 days was chosen as the time between doses.

Half of the animals in Experiment 2 were sacrificed and the other half maintained as a survival study group. The sacrifices were done systematically on a schedule to allow determination of radiation effects upon the hematopoietic systems. Each newt was weighed live and then killed by decapitation. In an effort to determine how severely the hematopoietic tissues were being damaged, red blood cell (RBC) counts were made to compare the concentrations of circulating erythrocytes in controls to the concentrations from single dose and two-

fraction groups. Blood for the RBC counts was drawn from the carotid arteries and diluted 1:100 with Hayem's Fluid. The RBCs were counted using a hemacytometer within 12 hours of sampling. These values are expressed as RBC/mm^3. This was calculated using the formula:

$$\frac{\text{number of cells counted x dilution factor (100) x 4,000}}{\text{number of small squares on hemacytometer counted (80)}} = \text{RBC/mm}^3$$

Both kidneys, the entire spleen, and the lower left lobe of the liver were dissected out and weighed wet. Any gross morphological changes were noted as indicators of damage to the hematopoietic or digestive systems.

Experiment 3 was designed basically the same as Experiment 2 except the fractionation time was lengthened to 15 days. No organ weight studies or blood counts were done with a study of recovery as reflected in MST values as the primary goal. To test whether the number of fractions made any difference on recovery a total dose of either 650 or 2000 rads was delivered in a single dose or two or four equal exposures. In this experiment, the source used was the Cobalt-60 gamma source rather than the x-ray source. The gamma source delivered only two-thirds the dose-rate (28 rads/min) of the x-ray machine (41 rads/min).

Experiment 4 was strictly a survival study to determine recovery under different conditions of dose fractionation and dose-rate.

RESULTS

The results of this study are presented in the order the experiments were conducted. Basic statements of observations and analyses of survival time curves will constitute the major portion of this chapter.

The mean or median survival times of the six groups of newts in Experiment 1 are listed in Table 2. This experiment was conducted to fill in the lower end of a general survival time-dose curve for Taricha granulosa. In an attempt to locate the lowest dose producing 100% lethality, the animals were held until their rate of death did not differ significantly from that of the controls. The 96- and 192-rad groups were definitely below this level. A dose of 288 rads was sufficient to produce 100% mortality. Since most of the animals were dead within 70 days post-irradiation (DPI), it can be assumed most of the deaths were a result of acute radiation damage. However, because these were spring collected animals, their deaths may have been accelerated due to the poor nutritional state they are in after winter starvation. Groups receiving 384 and 480 rads exhibited classic survival curves but without any apparent statistical difference in their MST values. A plot of these curves can be seen in Figure 1.

After the 48th day all newts that died were autopsied and any gross morphological changes noted. The most commonly noted changes were liver abnormalities (black color, mottled color, and blanching), heart defects (ventricular spots and engorging of the auricle) and atrophy and/or

Table 2 Effect of single doses of x-irradiation on mean survival times ± S.E. or median survival times of Taricha granulosa in Experiment 1

Radiation Dose (rads)	N	MST (days)
0	50	195
96	50	126
192	50	100
288	30	73 ± 8
384	30	49 ± 4
480	30	44 ± 2

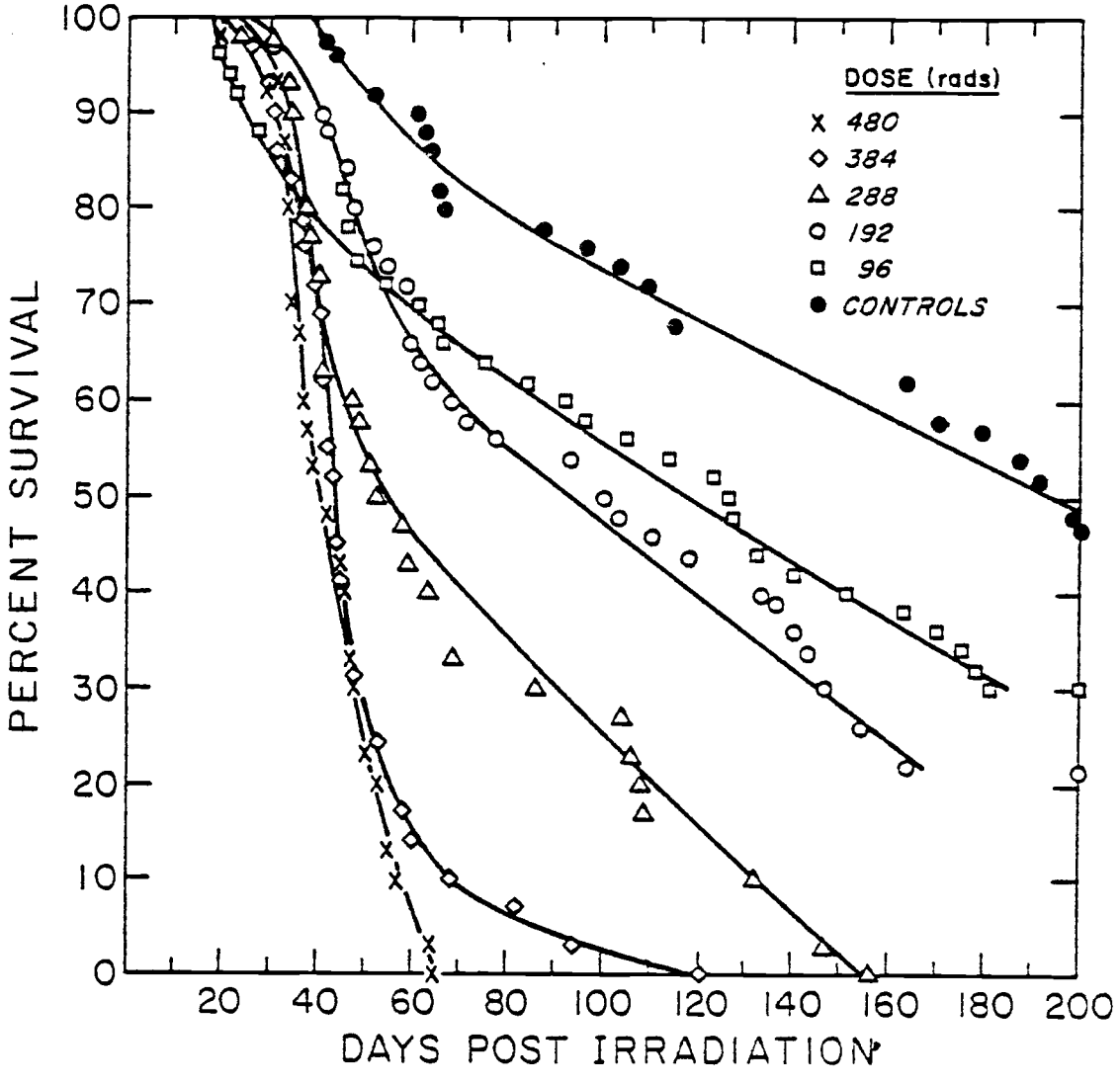

Figure 1 Survival of newts following doses of 0-480 rads x-irradiation in Experiment 1. Sample size was 30-50 animals per dose group.

discoloration of the spleen (blanching or darkening of the pigment). Percent frequencies of these observations are found in the Appendix. At least one of the three liver abnormalities was found in the majority of deaths. As liver abnormalities were also observed in a high percentage of control deaths, these conditions are probably not directly related to radiation damage. A peculiarity found in a small percentage of deaths in most groups was the retention of 2-10 ml of fluid within the visceral cavity. Spleen damage was shown primarily as shrinkage and in some cases a discoloration of the tissue. The majority of animals autopsied exhibited this effect. These effects and their relationships one to another are reported in Figure 2.

A systematic dissection of the animals in Experiment 2 was performed for the purpose of studying possible radiation damage upon the hematopoietic system. Organ weights and RBC counts were the major points of focus. Comparison of the concentrations of circulating erythrocytes between controls and fractionation groups in Experiment 2 proved to be inconclusive. Unexplained increases in the number of circulating RBCs after day 20 appeared in all groups. These results are listed in Table 3.

A study of organ weights in relation to dose was also conducted in Experiment 2. As seen in Figure 3, organ weights were normalized to percent of the live body weight of each animal. Relative kidney weight for all groups remained fairly constant for the duration of the study indicating no apparent damage. Relative liver weights in Figure 3 showed significant decreases as compared to controls at days 20 and

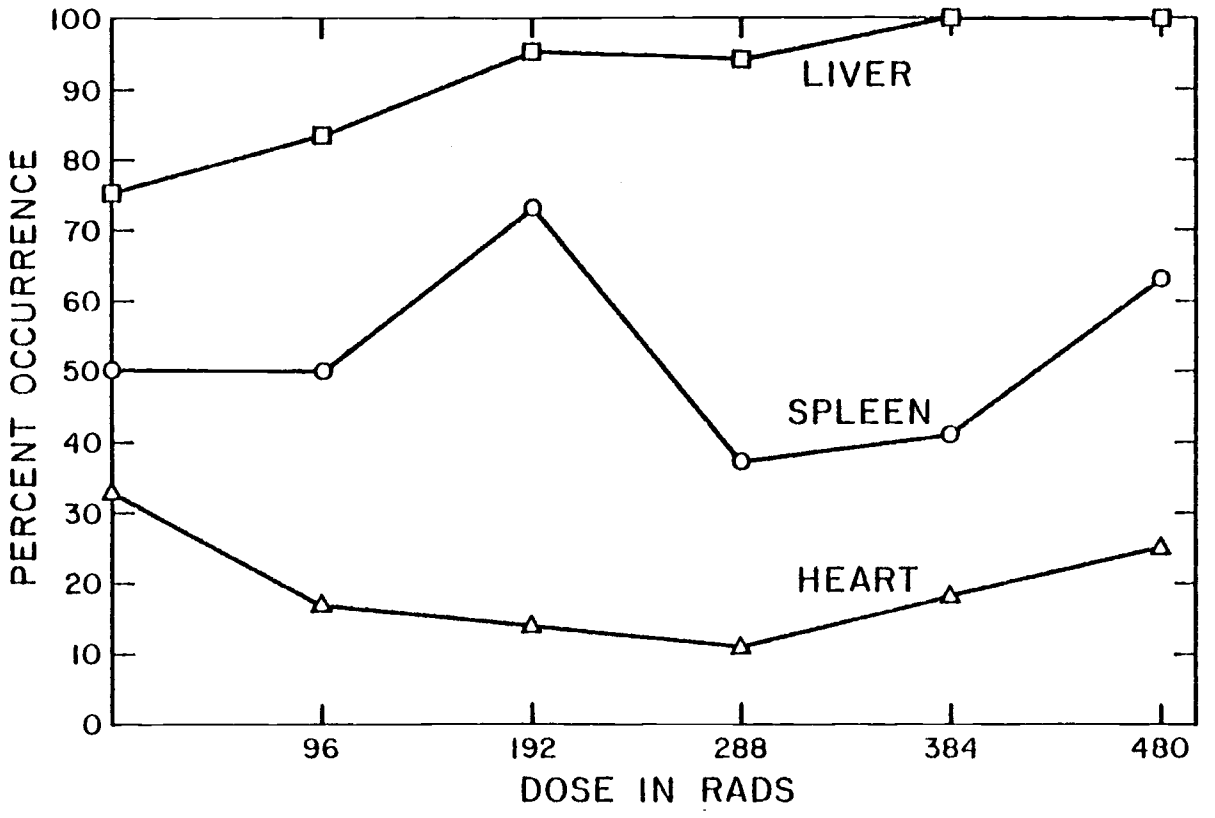

Figure 2 Relationships between the percent occurrence of gross morphological abnormalities in liver, spleen, and heart between dose groups of 0-480 rads as observed in autopsies performed in Experiment 1. Number of animals per point varies from 12-22.

Table 3 The means and standard errors of the circulating RBCs/mm^3 in the blood of newts after having received a total of 650 rads by either a single dose (650 rads) or two fractions (325 rads x 2) for Experiment 2. N = 7 or 8 newts for each sampling.

	RBCs/mm^3 x 10^{-3}		
	Control	Single Dose	Two Fraction
Sacrifice Day	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.
1	95 ± 20	115 ± 10	120 ± 15
3	135 ± 10	110 ± 10	135 ± 10
7	145 ± 10	140 ± 10	135 ± 15
9	125 ± 10	135 ± 20	120 ± 25
20	540 ± 30	545 ± 40	475 ± 40
45	450 ± 45	425 ± 50	445 ± 45

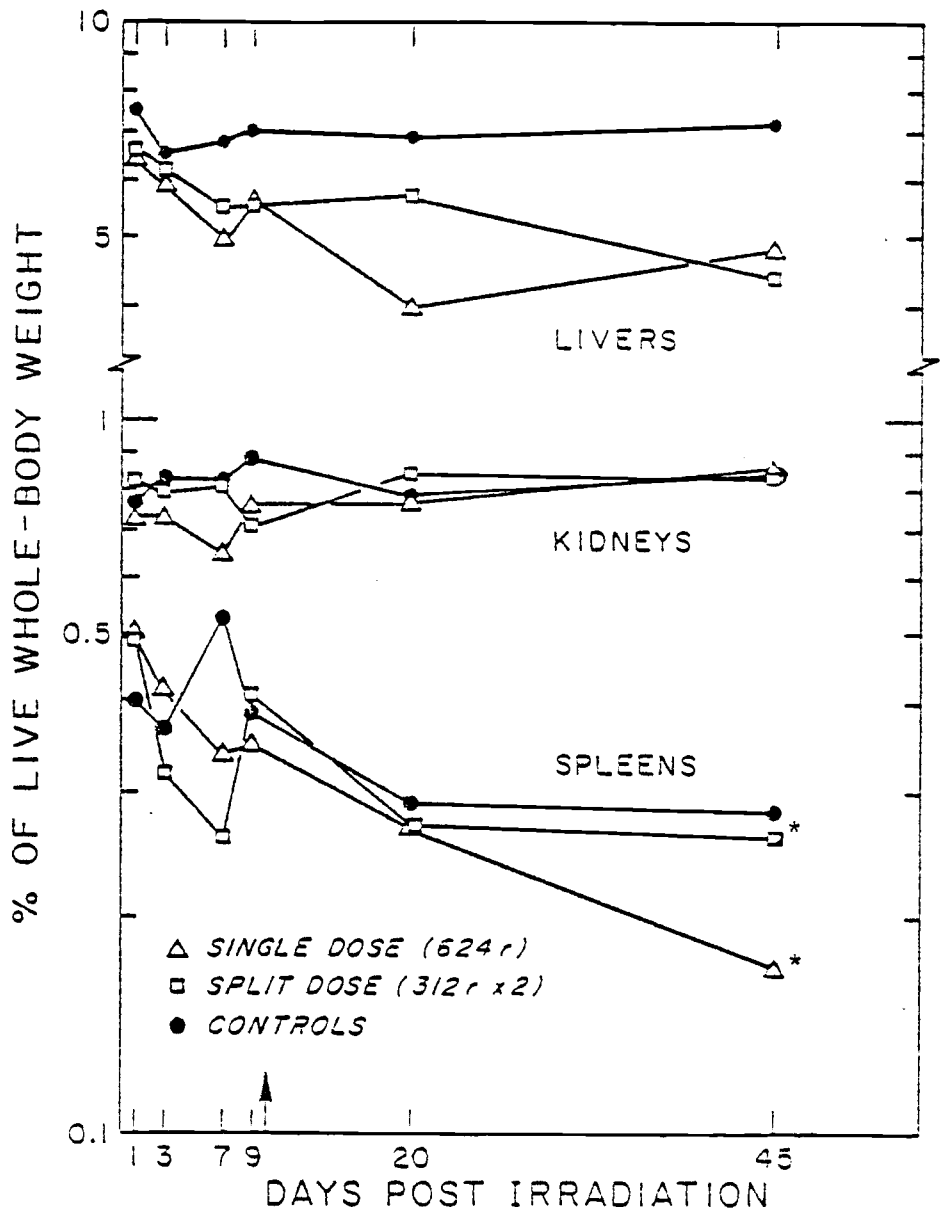

Figure 3 Comparison of relative organ weights of newts receiving 624 rads either as a single dose or as two fractions with organ weights of controls for evidence of hematopoietic damage. N = 8 animals except where an asterisk indicates 7. Arrows at day 10 indicates second half of split dose.

45 for both the single- and split-dose groups. A significant lowering of relative spleen weight could only be demonstrated at day 45 for the single-dose group. Reduction of relative irradiated organ weights to those of controls was determined to be indicative of radiation damage. Actual mean values and their standard deviations may be seen in the Appendix.

As evidenced by the survival curve in Figure 4, there was no statistical difference between survival times for single and split doses in Experiment 2. The median survival time for both one- and two-fraction groups was 45 days. This result led to the hypothesis that the fractionation time was too short and that I had observed merely an additive effect of the doses.

In view of the fact that an additive effect of the doses was observed in Experiment 2, Experiment 3 focused on survival studies using a longer fractionation time. Inspection of the survival curves in Figure 5 indicated a significant difference of survival times between all three dose administrations. Increasing the dose fractionation time to 15 days did indicate the presence of recovery. Again, the majority of the animals in all groups were dead by the 70th DPI with median survival times being 30, 45, and 52 days for the single dose, two- and four-fraction groups, respectively.

The design of single dose, two, and four fractions was also used to test for recovery using a total dose of 2000 rads in Experiment 3. As the survival curves in Figure 6 show, the MST values and standard errors were 29 ± 7 days for the single-dose group, 30 ± 2 days for two

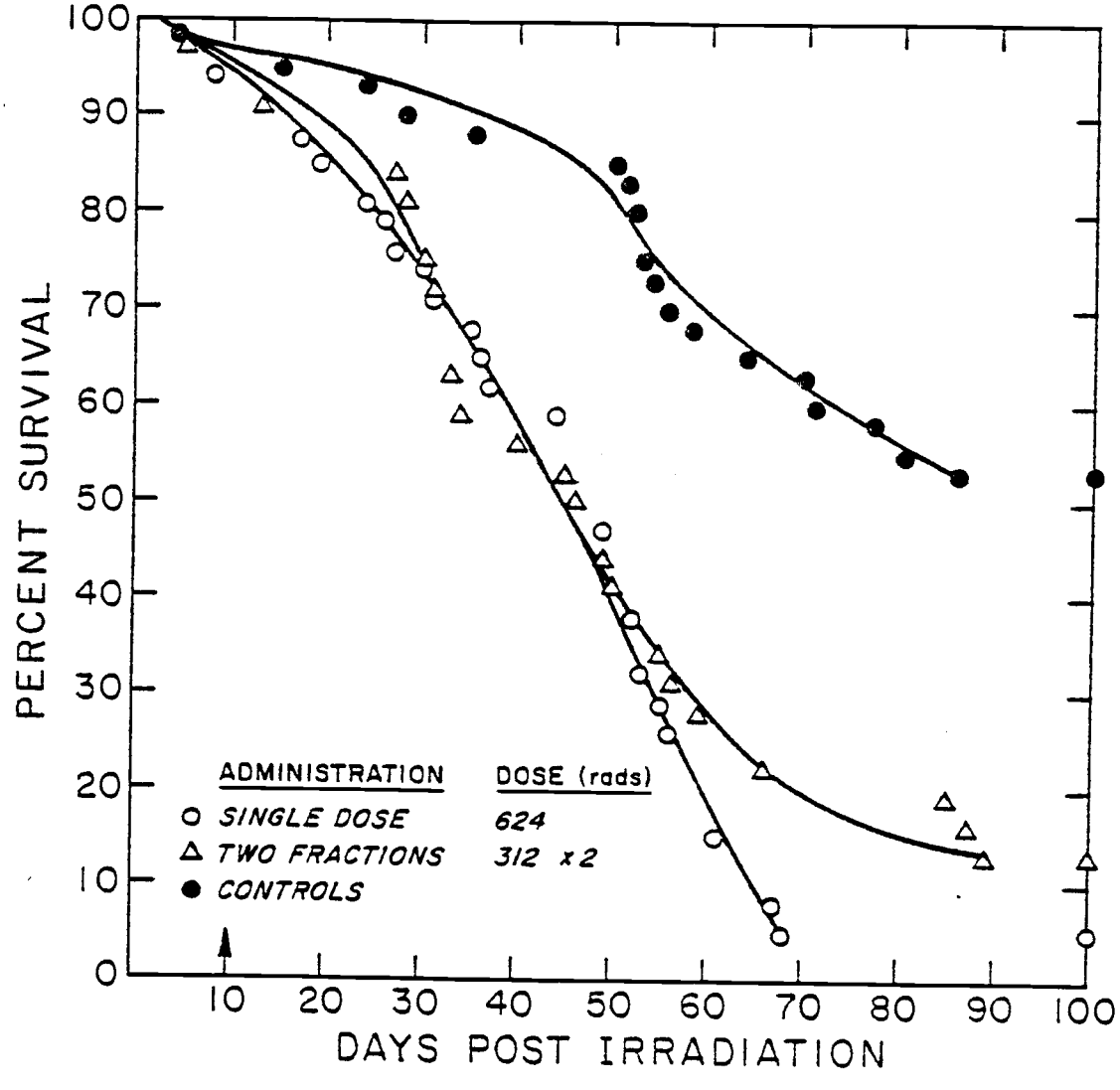

Figure 4 Survival times of newts in Experiment 2 after receiving 624 rads x-irradiation as a single dose or as two fractions with a 10-day fractionation interval. Sample size was 32-40 animals per dose group. Arrow indicates second half of split dose.

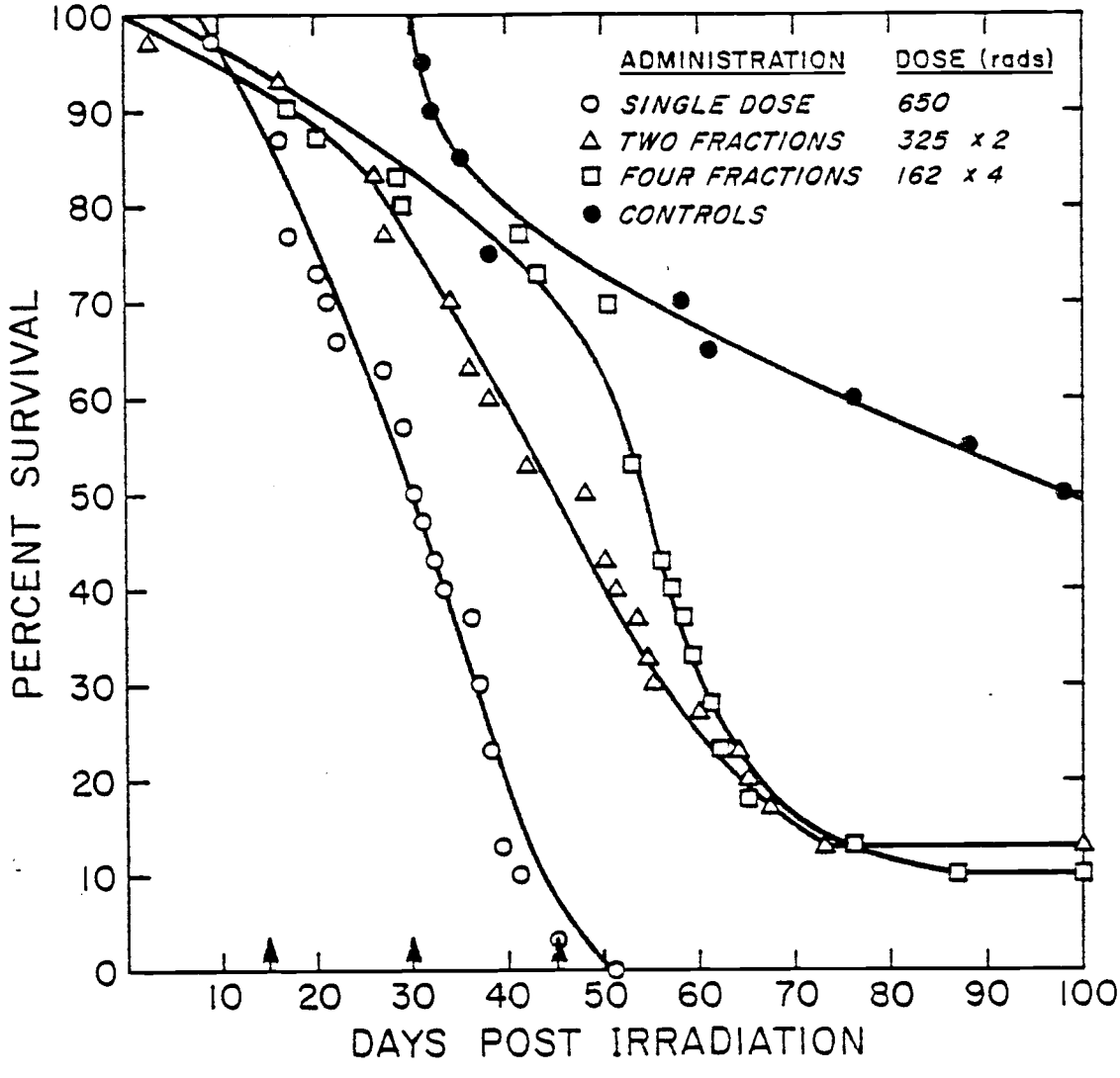

Figure 5 Survival times of newts in Experiment 3 after receiving 650 rads gamma irradiation as a single dose or as two or four fractions at 15-day intervals. (Arrows indicate subsequent dose fraction administrations.) Sample size was 30 animals per dose group.

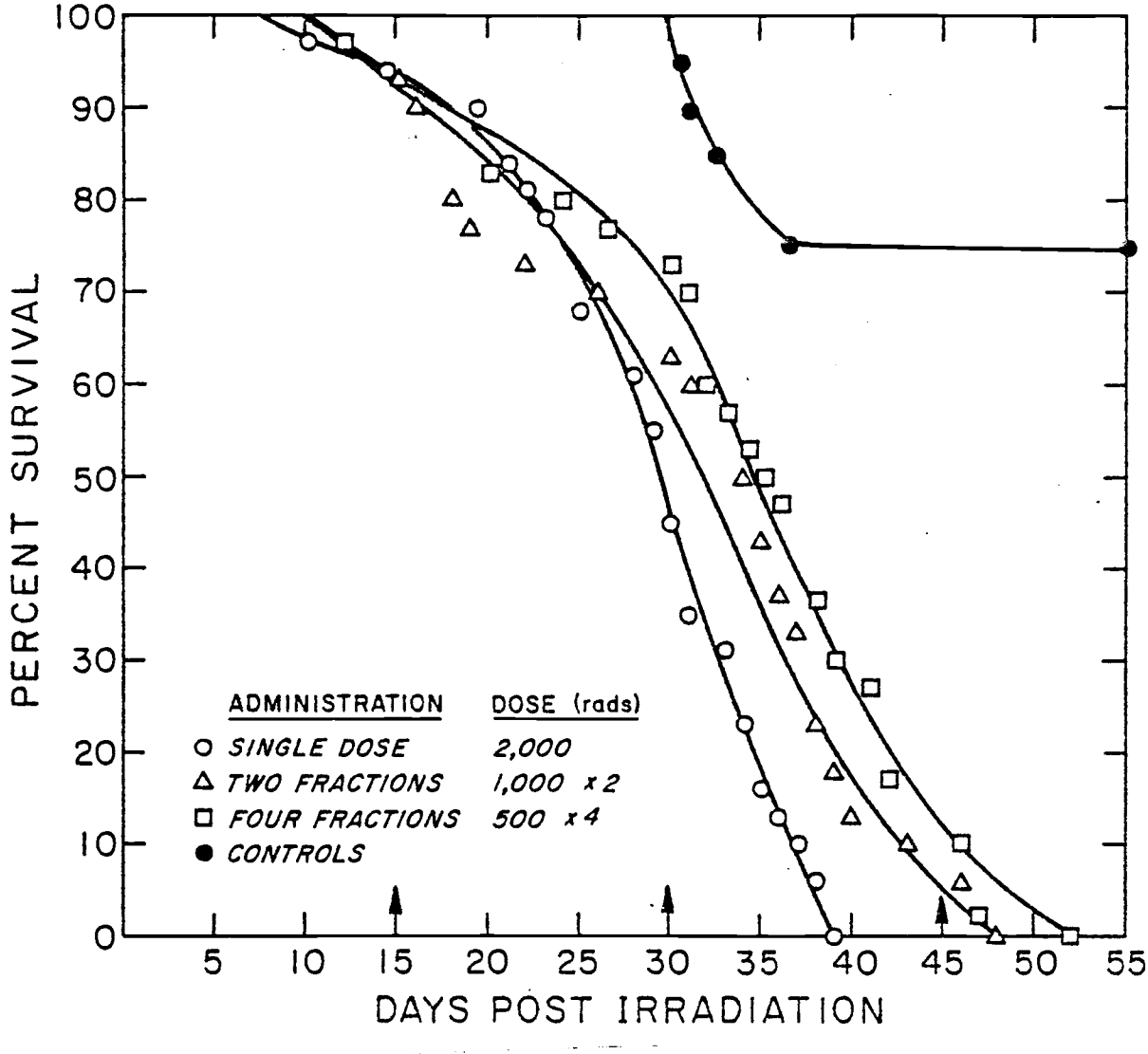

Figure 6 Survival times of newts in Experiment 3 after receiving 2000 rads gamma irradiation as a single dose or as two or four fractions at 15-day intervals. (Arrows indicate subsequent dose fraction administrations.) Sample size was 30 animals per dose group.

fractions, and 35 ± 2 days for the four-fraction group. All irradiated animals had died by the 55th DPE.

Experiment 3 also tested dose-rate effect between the xray source (41 rads/min) and the gamma irradiator (28 rads/min). The survival times for 624 rads in Experiment 2 and 650 rads in Experiment 3 can be seen in Figures 4 and 5. When the dose was given in two equal fractions there was no difference in median survival times in each case (46 DPE). However, there does appear to be an unexplained shortening of survival time (30 DPI) at the lower dose-rate (28 rads/min) in Experiment 3 for a single dose application while the single dose survival time (44 DPI) remains approximately the same at the higher dose-rate (41 rads/min) in Experiment 2. This was not expected.

Toe-clipping is a fairly well accepted means of animal identification. It was noted at this time that it is not advisable to add this trauma to an already radiation-traumatized newt. Control animals did not respond adversely to the procedure. However, when animals which had previously received the first fraction of radiation (182 rads) were toe-clipped, they all responded with the release of massive amounts of neurotoxin from skin glands. Out of a group of twenty, several went into shock and three deaths occurred.

Experiment 4 used the same design of single dose, two, and four fractions to deliver a total dose of 10,000 rads to all experimental groups. However, because all the experimental animals had died the fourth fraction was never given. Tests for an obvious dose-rate effect

were made using the two dose-rates available for the gamma irradiator (28 rads/min and 1150 rads/min).

As evidenced in Figures 7 and 8, there were no significant differences between the survival times of the two dose-rates for the two- and four-fraction groups. The single-dose group for the low dose-rate (28 rads/min) showed a significantly shorter survival time than the corresponding high dose-rate group (1150 rads/min). This was totally unexpected.

There was, however, a significant difference at the .05 level between the survival times of the single-dose and four-fraction administrations within each of the dose-rate groups. The MST values and standard errors for the single dose, two- and four-fraction doses, respectively at 28 rads/min, were as follows: 29 ± 0.7 days, 27 ± 2 days, and 33 ± 2 days. The MST values and standard errors for the single-dose two- and four-fraction doses at 41 rads/min expressed in days are as follows: 28 ± 1, 28 ± 1, and 35 ± 1.

It was noted that nearly all the animals receiving more than 650 rads developed "light patch" formation sometime before death. This condition as described by Lappenbusch (1969) was evidence of depigmentation of the skin. The condition persisted until death in all cases. Higher doses (2500 rads to 10,000 rads) resulted in primary or secondary lesions, also discussed by Lappenbusch (1969), in some of the animals in addition to the "light patch" formation. A severe phalangeal ulceration and erosion, often with distal cartilages exposed

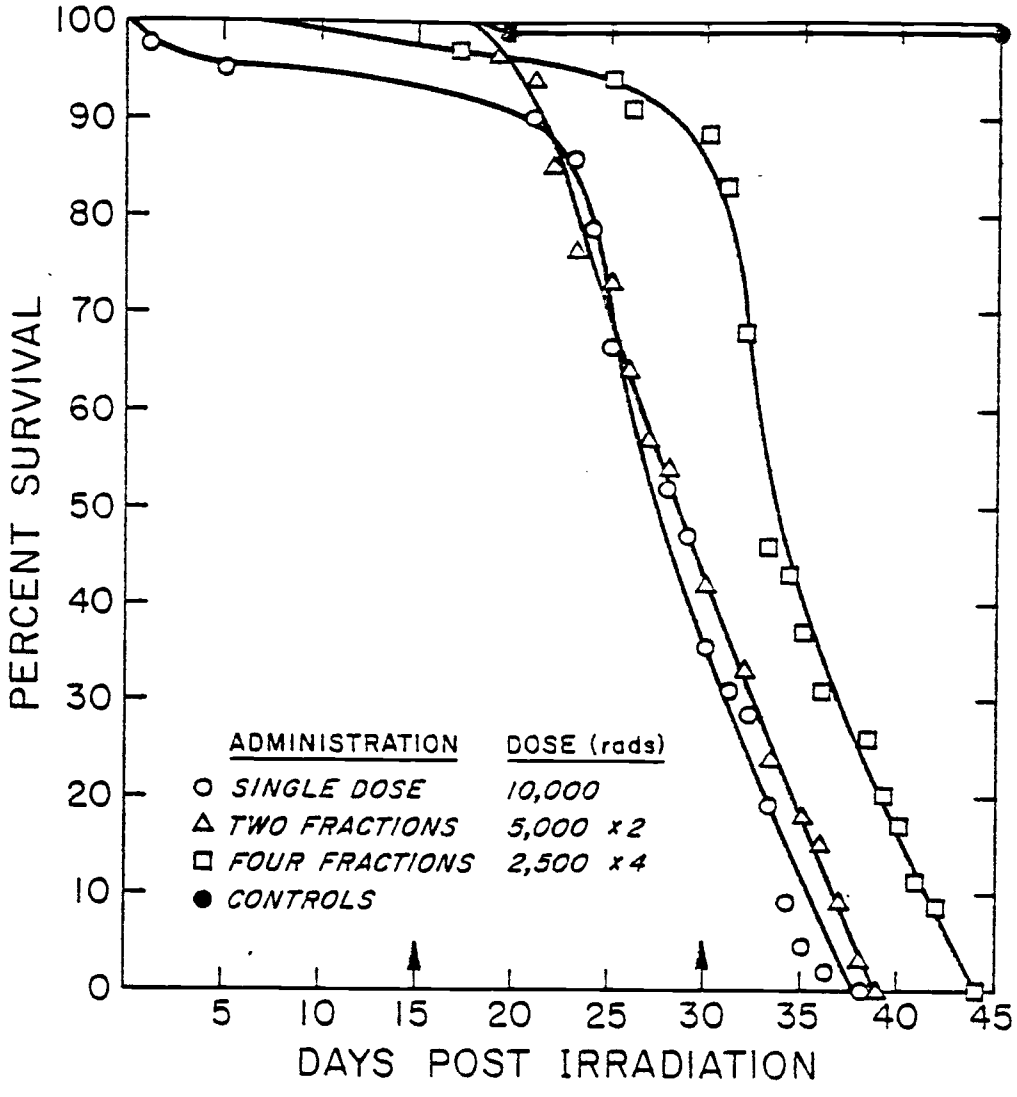

Figure 7 Survival times of newts in Experiment 4 after receiving 10,000 rads gamma irradiation as a single dose or as two or four fractions at 15-day intervals at 28 rads/min. (Arrows indicate subsequent dose fraction administrations.) Sample size per dose group was 33-42 animals.

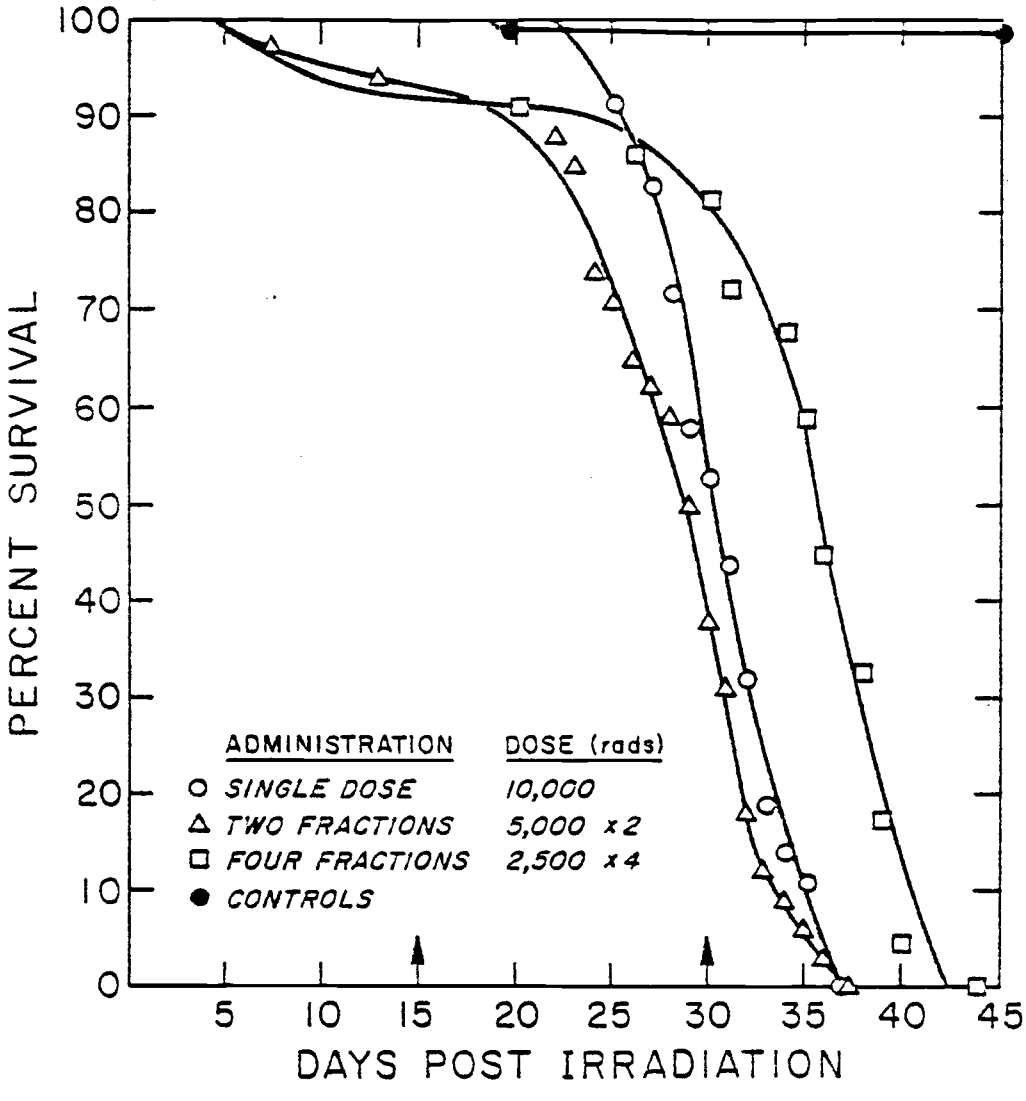

Figure 8 Survival times of newts in Experiment 4 after receiving 10,000 rads gamma irradiation as a single dose or as two or four fractions at 15-day intervals at a dose rate of 1150 rads/min. (Arrows indicate subsequent dose fraction administrations.) Sample size per dose group was 22-36 animals.

was noted after about 100 days in animals receiving doses of 2000 rads or more.

DISCUSSION

In an effort to gain an understanding of recovery in the newt, Taricha granulosa, results shown as survival time curves, organ studies, dose-rate effects, and skin damage will be discussed in such a manner as to interrelate the four experiments. As mentioned before, recovery from radiation damage in amphibians achieved by fractionated doses has been a relatively unexplored field of study. Extensive work with mammals (Mole, 1956; Storer, 1964; Corp and Mole, 1966; Ben-Hur et al., 1974) and goldfish (Egami and Etoh, 1966; Egami et al., 1967; Etoh and Egami, 1967; Egami, 1969; Hyodo-Taguchi, 1970) had indicated what might be expected if amphibians followed the same patterns of recovery as these classes of vertebrates.

Survival Time Studies Through Dose Fractionation

Mean or median survival time was the primary criterion of comparison from one experiment to another and between dose groups within experiments in this study. The presence of a significant difference in MST values between two groups receiving the same total dose indicated the existence of recovery. This is in support of the hypothesis that recovery can be shown by fractionating the dose. Elkind and Sutton (1959) showed with mammalian cell cultures that daughter cells did not inherit the same amount of damage as the parent cell had received. As evidenced by the daughter cells, all or part of the original damage

had been repaired prior to mitosis. Those cells which did not reproduce or had died must have reached their threshold of sublethal accumulated damage after which point deaths begin to occur. When the same total dose was administered in two parts separated by some interval of time it was found to be less effective in causing cell-killing than when given all at one time (Hornsey and Vatistas, 1963). The shoulder of a survival curve is a level area at the beginning of the curve where no visible evidence of radiation damage can be seen. It indicates the presence of damage accumulated toward the threshold lethal dose at which point deaths began to occur (Elkind et al., 1967; Lappenbusch, 1969). Up to the threshold of accumulated damage causing lethality the animal has retained the ability to repair that damage or with the addition of more irradiation to exhibit a lethal response.

Lappenbusch (1969) found the threshold lethal dose for Taricha granulosa to be about 200 R (192 rads). Sparrow et al. (1970) had predicted from nuclear and chromosomal values that the LD_{50} was $\sim$ 170 rads. In Experiment 1 radiation deaths were found for all doses including the 96 rad group. Doses of 96 and 192 rads were not large enough to cause death in 100% of the individuals and observations for these groups were terminated when the rate of death was the same as that of the controls. Doses of 288, 384, and 480 rads produced death in all the individuals as would be expected. The animals used in Experiment 1 were collected during the late winter at which time they have little stored fat after being in their terrestrial phase for several months. This part of their seasonal cycle has been termed the "inactive" phase by Algard et al. (1974).

Spring and summer, conversely, are termed their "active" period during which time they have recouped physically. Animals in the "inactive" phase exhibited a shorter survival time than those in the "active" phase due to their poor nutritional status at the outset. The slope of the control deaths was steep and nearly paralleled that of the 96-rad group. It is clear that more work needs to be done to better define the LD_{50} for Taricha granulosa at 20°C.

As stated earlier a significantly longer MST value was interpreted as evidence of recovery between fractions with the same total dose as compared to a single dose. In all experiments where the dose was fractionated into four parts there was marked increase in survival time. Splitting a dose into two fractions did not uniformly result in significant differences in MST, however. Splitting the doses of 10,000 and 650 rads increased the MST significantly at the .05 level or better. Splitting 2000 rads suggested a strong trend towards a statistical significance which could not be supported at the .05 level. Splitting the 624 rad dose did not lengthen the survival time at all since the fractionation interval was too short.

Survival times were measured from the day of the first administration of any dose. It must be taken into account then, that in the cases of the 2000-rad and 10,000-rad groups, the majority of deaths at these supralethal doses had occurred prior to the final fraction in the four fraction groups. While it would be impractical for supralethal doses, it it suggested that in the future another point be chosen as time zero for determination of MST. The Japanese began measuring

their fractionation survival times after the last fraction had been given (Egami et al., 1967). After the last fraction does not seem to be an adequate choice because deaths generally have begun to occur prior to time zero. Designating time zero as the point at which any of the organisms received any radiation is inadequate also because there are few organisms that have received the same total dose at any time for comparison until after the last fraction has been given. A mid-point in time of the fractionated doses may offer the best solution. This is a problem of fractionation experiments which does not lend itself to an easy solution and makes fractionation studies by different workers difficult to compare.

Primary interest in the study of recovery had been shifted to the cellular level and to the radiosensitivity of the cell during different phases of its cell cycle (Elkind et al., 1965; Egami, 1969; Hyodo-Taguchi, 1970). Knowledge of the modulation of recovery during the cell cycle makes it possible to determine when to apply subsequent fraction(s) of radiation to the system in order to achieve either the most protection or effectiveness, depending on the experimental objective. If an improper estimation is made as to when an organism's most radiosensitive period occurs, for the application of the next dose, the result can be a lack of recovery. This is shown as a simple additive effect of the doses as though they were a single dose, when the potential for recovery was actually there. Recovery rate was originally assumed to be a simple exponential function of time. Mole (1956) suggested that this was not generally the case. Subsequently, Corp

and Mole (1966) proposed that recovery was not an exponential curve at all, but an initial period of rapid recovery followed by a linear segment of slower recovery. This was supported by Holloway et al. (1968) from their work with hamsters. They stated that recovery was non-exponential.

In fractionation studies the choice of determining the fractionation interval is critical and can be affected by several conditions. An error in determining the shape of the recovery curve (i.e., whether there exists a linear segment) or neglect of a factor that may affect a cell's progression through its cycle could cause a wrong fraction interval to be chosen. In Experiment 2 assuming that there were no unaccounted for factors, that the recovery curve for this newt was primarily linear and using Filipy's (1977) estimate of intestinal crypt cell cycle time, I computed a fractionation time of 10 days at 20°C. As Figure 4 shows, a merely additive effect of dose was observed in this experiment. Evidently an incorrect assumption had been made as to the shape of the recovery curve. Fractionation time was increased to 15 days and, as seen in Figure 5, this was adequate to allow recovery.

The four main factors that affected these experiments were those of temperature, fraction interval, number of fractions, and fraction size. A fifth factor of undetermined importance was the nutritional state of the different experimental and control groups. Two experiments were conducted with well-fed summer animals and two with starved winter animals. I manipulated this factor by attempted feedings but

this too was of undetermined value. Temperature had been shown to be a very important factor affecting the occurrence and rate of recovery in both mammalian cells (Belli and Bonte, 1963; Elkind et al., 1965; Ben-Hur et al., 1974) and goldfish (Egami and Etoh, 1966; Egami et al., 1967; Etoh and Egami, 1967; Egami, 1969). The shapes of the survival curves for Taricha granulosa showed a similarity to those of mammals, a result which Egami et al. (1967) also noted for Oryzias latipes.

A common effect noted in both the mammalian cell cultures and fish was that the effect of fractionation on lethality was smaller when the animal was kept at lower than normal temperatures during the interval between fractions (Etoh and Egami, 1967; Szechter and Schway, 1977). Hyperthermia, on the other hand, caused an enhancement of radiation response above 37°C in rodents (Ben-Hur et al., 1974). This enhancement may have stemmed from an inhibition of repair of sublethal damage as well as an enhancement of lethal damage. They felt this enhancement effect may have been possible because there appeared to be a delay in the redevelopment of a normal capacity for sublethal damage. The shoulder of a survival curve is a flat area at the beginning of the curve during which sublethal damage accumulates. When the curve drops towards the base line deaths occur. Redevelopment of this shoulder for accumulated damage is an expression of repair of sublethal damage already in the system (Elkind et al., 1967).

As early as 1948, Patt and Swift proposed that an altered sensitivity at low temperatures was apparently due to a decrease in the rate of development of radiation damage rather than to any appreciable

recovery. Later, a reduction in radiosensitivity by a type of "protection" rather than an enhancement of recovery was what Belli and Bonte (1963) felt explained reduced deaths at lower temperatures. This "protection," although not well understood, was thought to be closely associated with the slowed cell metabolism and mitosis and the recombination of molecules before deleterious reactions could take place. Szechter and Schway (1977) indicated that repair at lower temperatures is less rapid than at 37°C and remained incomplete with slow repair unable to account for all of the damage.

The idea that recovery at low temperatures is inhibited is supported by the work of Egami and Etoh (1966) and Etoh and Egami (1967) who showed that with Oryzias latipes the rate of recovery slowed under low temperature. Also animals held at a low temperature lived longer until raised to a higher temperature at which they died at the same rate as animals irradiated and held at the higher temperature (Hyodo, 1965). From this there appeared to be evidence that neither recovery nor radiation damage manifestation took place until the temperature returned to normal (Allen et al., 1951; Berry and Oliver, 1964).

The number of fractions did seem to be an important factor in the survival of Taricha granulosa in the present study. As mentioned before some degree of significance between the survival times of the two- and four-fraction groups occurred providing all the experiments had the first two qualifications of optimum temperature and fractionation interval times met. This is in contrast to Kohn and Kallman (1957) who found no fundamental change in the distribution of deaths with time

by fractionation using mice. Brown et al. (1960) and Etoh and Egami (1967) felt that while fractionating the dose lengthened survival time, the number of fractions was of no consequence. It was the total time from the beginning of the irradiation to the end of all the fractions that was important. A possible explanation for this discrepancy is that it was difficult to compare work between laboratories when it was not specified whether the fraction number was varied within one constant amount of exposure time or whether the fraction number increase also was reflected in an overall exposure time increase.

The size of the fraction, as it related to the rate of recovery, is a variable which has not been well studied. Storer (1961) felt that the rate of recovery showed a marked dependence on size of the initial dose, with larger doses repaired more slowly. Repair rate, based on percent repair, decreased inversely with the square of the dose. Therefore, the fraction of latent damage repaired, as measured by his technique, decreased progressively with increasing dose. Mole (1956) also felt that the initial recovery rate varied inversely with the size of the initial dose. Etoh and Egami (1967) found with Oryzias latipes that the effects were more noxious if the initial dose given was the larger of the two doses. This may explain why the amount of recovery increased with decreasing initial dose in my fractionation experiments. For example, the 10,000 rad group survived for a shorter time than did the group which received 5,000 rads initially and another 5,000 subsequently for the same total.

Sparrow et al. (1970) predicted the LD_{50} for Taricha granulosa to be ∿ 170 rads. Most estimates range from 100-250 rads (Lappenbusch and Willis, 1970; Willis and Lappenbusch, 1976). In comparison to the $LD_{50/30}$ values for man (400 rads) and mice (550 rads) the LD_{50} value for the newt is low. Fractionation intervals are computed on cell cycle recovery times generally. Newts have a long cell cycle time and hence, a slow rate of recovery (Filipy, 1977). Animals with lower LD_{50}s according to Kohn and Kallman (1957) and Leong et al. (1964) have a slower rate of recovery. This affects their systemic recovery and latent times. Amphibians appeared radioresistant in early studies (Patt and Swift, 1948) when in reality they just have long cell cycle times, slow recovery rates and long latent times.

Organ Weight Studies and Hematopoietic Effects

Random autopsies of deaths of animals in all irradiated groups in Experiment 1 led to the hypothesis that gross morphological abnormalities were symptomatic of radiation damage. Random control deaths were autopsied also and showed a smaller incidence of similar abnormalities. While this is not understood, feeding prior to death in these animals was sporadic and a possible cause of death and organ damage could be linked to malnutrition.

Since the random organ studies in Experiment 1 had indicated possible radiation effects upon the liver and spleen, Experiment 2 was designed to present a controlled study of these organ responses with

time. It was noted upon systematic dissection that there were significant differences at days 20 and 45 in the relative liver weights in both single dose and the two-fraction groups and the controls. The single-dose group showed a significant reduction in relative spleen weight from controls at 45 DPI. Algard et al. (1974) in their work with Taricha granulosa, also noted such abnormalities of the spleen and liver. It was known that, because of the lack of long bone marrow in urodele amphibians, the spleen was the site of erythropoiesis (Brunst, 1958b) and the liver the site of granulopoiesis.

The RBC counts of Taricha granulosa are subject to cyclic annual patterns as found by Friedmann (1974) under normal conditions. Animals maintained at 20°-24°C showed an average of 112.7 ± 35.6 x $10^3/mm^3$ of circulating RBCs. As shown in Table 3, the values for circulating RBCs for the first four sacrifice dates were in agreement with those values listed by Friedmann. However, for days 20 and 45 there is an unexplained dramatic jump in the values of circulating RBCs. While this was totally unexpected and not understood, there does seem to be a roughly corresponding drop in spleen weight at day 45 for the single-dose group which may signal a "dumping" of erythrocytes into the vascular system. It was also observed that some of the animals had definite fluid loads in the visceral cavity upon dissection which could be taken as evidence of an osmo-regulatory malfunction resulting in fluid being lost from the vascular system. If this was the case it would cause what appeared to be an increase in the number of circulating RBCs while actually merely increasing their concentration. One or both of these conditions may be a

partial explanation for this unexpected rise in RBC concentration. Unaccountably, the same rise was noted in the controls.

Dose-Rate Effect

Brown et al. (1960) have maintained that the radiation dose needed to kill mice depends on the overall exposure time; i.e., from the beginning of the first fraction to the end of the last. They and the Japanese workers using fish also concluded that dose-rate and the number of fractions seemed to have no effect upon the survival time of their organisms. Etoh and Egami (1967) pointed out that fractionation experiments with Oryzias latipes had shown a lack of recovery if the organism was kept at a low temperature between fractions. The result was an effect as though the doses were cumulative. Despite this evidence it is well-accepted that a single dose is more effective than either fractionated or chronic irradiation in producing cell death and, hence, death of the organism. According to Etoh and Egami (1967) repair appeared to proceed during radiation-on time during chronic irradiation as well as during the intervals between fractions.

Temperature dependence seems to be the primary factor affecting repair in a cell and, hence, an organism. This is particularly true of poikilotherms which lack the innate body temperature control of the homeotherms. Low temperature, by depressing cellular activity, seems to retard or completely inhibit the recovery process, while hyperthermia seems to enhance the radiation response resulting in a greater number

of deaths. Dose-rate and the number of fractions appear not to have any consequences upon the survival time if the temperature is not optimal for regular cellular activity. For Taricha granulosa, Willis and Prince (1967) and Willis and Gruber (1975) showed that single dose radiation survival times are markedly different when tested at 10° and 20°C (Figure 9). In the current experiments on this organism at 20°C the number of fractions did appear to be significant to the length of survival time. Four fractions gave a significantly longer survival time than either a single dose or a two fraction administration in the groups totaling 10,000 and 2,000 rads. As previously mentioned, the four fractions of the 650-rad group totaled more than 650 rads due to a calculation error at the time of irradiation. However, this slightly higher total dose (728 rads) also had a significantly longer MST value as compared to its single-dose or two-fraction groups' MST values. This is in contrast to Kohn and Kallman (1957) who did not observe that fractionation caused a fundamental change in the distribution of deaths in mice. The number of fractions seems then to be the second most important factor in the repair process after an optimum temperature for cellular progression.

Despite the fact that there was a dramatic difference between the dose-rates available with the gamma irradiator in Experiment 4 (28 and 1150 rads/min) there was no significant dose-rate effect between the two dose-rates in either the two- or four-fraction groups. There did, however, appear to be a significant lengthening of the single-dose group survival time of the 10,000 rad (1150 rads/min) group over the

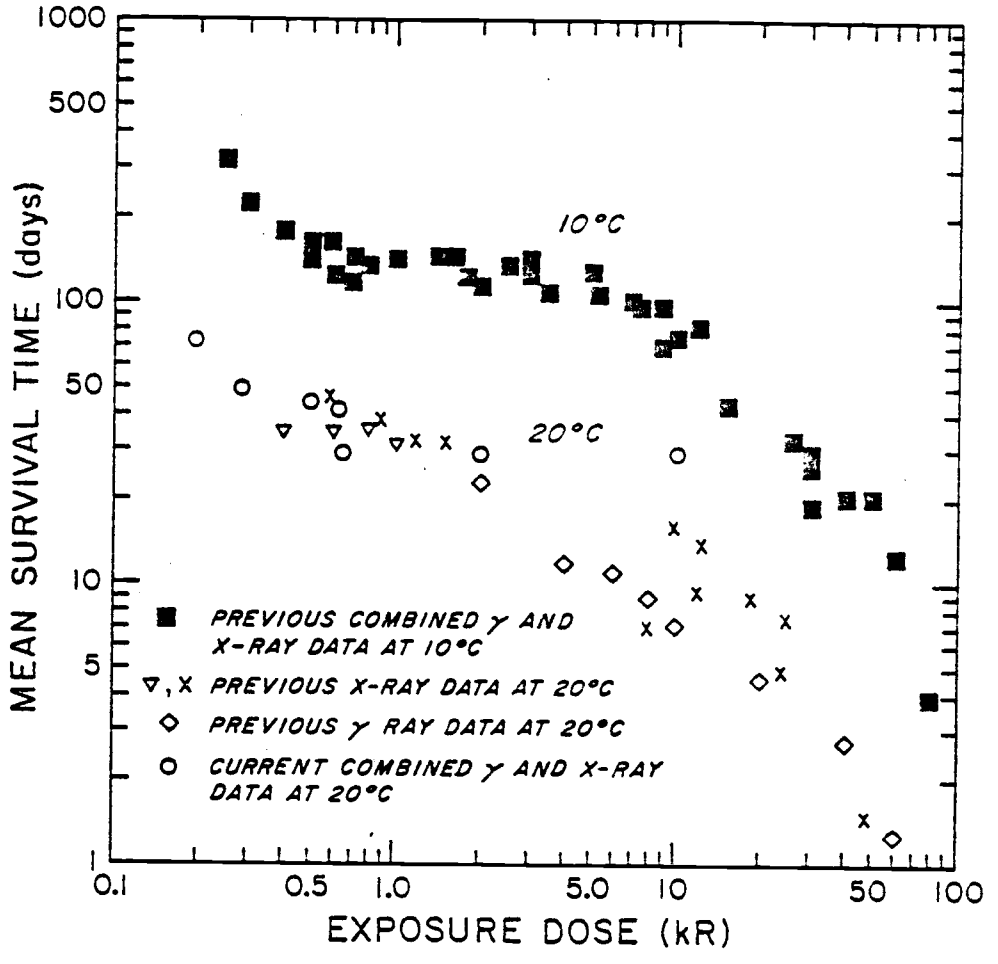

Figure 9 Survival time-dose curves for previous and current work in this laboratory with Taricha granulosa. This includes values for both 10° and 20°C after doses of gamma or x-irradiation.

10,000 rad (28 rads/min) group. Experiments 2 and 3 allowed comparison of the dose-rates of the x-ray irradiator (41 rads/min) and the gamma irradiator (28 rads/min), respectively. It was found that while the two-fraction MST values were not significantly different, the MST value for the 624-rad group (41 rads/min) was significantly longer than that of the 650-rad group (28 rads/min). In both of these cases the groups receiving the single dose at the higher dose rate survived significantly longer. This was not expected and while dose-rate effects have not been shown before it would seem logical for the converse to be true.

A possible explanation is that in the second case it must be taken into account that the animals in the single dose group of 650 rads were from the "inactive" phase as defined by Algard et al. (1974) and, when in that state, are known to have a markedly decreased survival time. However, this does not account for the difference at 10,000 rads as these animals were from the same collection. I would like to suggest that such a rapid delivery of a supralethal dose may not allow the cells to express the full extent of the radiation damage possible from the administration. Even though 10,000 rads were absorbed, the organism survived as if it had received a lower dose. While this is not understood, it might be attributable to recombination of damage-causing free-radicals. There might also be a shortage of molecules available for free radical production at the time of the dose.

It then seems that temperature is of primary importance to survival followed by the number of fractions. The question of whether dose-rate

has any effect upon survival time can not be conclusively answered from this investigation.

Skin Damage

One of the most obvious effects of radiation damage in Taricha granulosa is necrosis of the epithelium. Damage to the epithelium was noted by Brunst (1958a) in the adult axolotl (Siredon mexicanum) and interpreted as a major cause of death. The skin plays an important respiratory role in various salamanders and newts. Damage to the epithelium must be assumed to interfere with this function. In the common European newt, skin vascularity is responsible for 74% of the respiration. Smith (1967), in a study of the respiratory ecology of Taricha granulosa, noted the skin seemed to function year around in carbon dioxide release and heavily in the winter (and less heavily in the summer) for oxygen uptake. Lappenbusch and Willis (1970) felt that the skin lesions in Taricha granulosa they observed were probably not just from local irritation of the skin but from stress or damage to other organ systems as well.

"Light patch" formation, as defined by Lappenbusch (1969), was the mildest form of skin damage. This was evidenced as a lightening of the pigment at all protruding dorsal points, such as the ridge of the tail, spine, elbows, and knees, Close to 75% of all the animals receiving from 288-10,000 rads experienced some lightening along these stress points before death. There was also the formation of open sores defined

by Lappenbusch and Willis (1970) by severity as primary and secondary lesions. These were visible in groups receiving doses in excess of 1000 rads. Wounds such as these persisted until death. Brunst (1958a) in Siredon mexicanum considered skin lesions primary cause of death. Algard et al. (1974) found wound healing greatly reduced in Taricha granulosa after irradiation. My results support this. This is in contrast to Brunst et al. (1953) and Jakowska et al. (1958) who maintained that amphibians, unlike mammals, could continue phagocytic activity after irradiation and that active granulopoiesis did take place. Thus, they felt bacteremia was absent at all stages after irradiation. As evidence against that hypothesis, Algard et al. (1974) observed a severe phalangeal erosion frequently with the distal cartilages protruding in groups held more than 90 days. I observed the same effects. In this laboratory in Experiment 4 earlier than 90 days it was observed that any minor abrasion became easily infected. Once an irradiated animal developed this condition it was not able to overcome it and the condition persisted until death. Friedmann et al. (1970) and McCurdy et al. (1974) also observed this in this species in their laboratories.

SUMMARY AND CONCLUSIONS

This study has shown that recovery from radiation injury does take place in Taricha granulosa. From the results presented here it seems reasonable to conclude that recovery is an event which can be affected by various factors. In order of importance these are temperature, dose fraction interval, and fraction number. It appeared that the size of the initial dose fraction and the LD_{50} affected the rate of recovery. Dose-rate effect could not conclusively be determined in this study. It may be that even the lowest dose-rate used (28 rads/min) was too high to allow expression of a dose-rate effect, if such a phenomenon exists in newts.

I would suggest in the future that survival studies be done with newts all of one sex from either the "active" or "inactive" phase, but not both. This would eliminate the differences in survival times we have observed due to their nutritional state.

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APPENDIX

Table 1 Analysis of the natural well water (Oregon State University Oak Creek Fisheries Laboratory, Corvallis, Oregon) and the synthetic pond water (Amphibian Ringer's Solution diluted to one-tenth standard concentration) used in the present series of experiments. The analysis was conducted by the Laboratory Services Branch of the National Environmental Research Center, Pacific Northwest Environmental Research Laboratory, Corvallis, Oregon, on 18 May 1974.

Test	Well Water	Synthetic Pond Water
pH	7.8	6.9
Total inorganic carbon	24.0 mg/l	2.0 mg/l
Alkalinity	106.0 mg/l	15.0 mg/l
Bicarbonate alkalinity	106.0 mg/l	15.0 mg/l
Total hardness	90.0 mg/l	32.0 mg/l
Calcium	28.0 mg/l	6.6 mg/l
Magnesium	6.4 mg/l	0.2 mg/l
Sodium	9.0 mg/l	270.0 mg/l
Potassium	1.0 mg/l	8.2 mg/l
Chloride	4.0 mg/l	384.0 mg/l
Total copper	5.0 µg/l	31.0 µg/l

Table 2 Percentage occurrence of gross morphological abnormalities for the six dosage groups of T. granulosa in Experiment 1.

Dose (rads)	0	96	192	288	384	480
N =	12	12	22	19	17	16
Percentage of abnormalities in autopsies						
Liver						
Black	33	17	31	26	40	31
Mottled	9	33	18	42	25	38
Blanched	33	33	36	26	35	31
Total Liver Abnormalities	75	83	95	94	100	100
Heart						
Abnormal	33	17	14	11	18	25
Normal	67	83	86	89	82	75
Spleen						
Abnormal	50	50	73	37	41	63
Normal	50	50	27	63	59	37
Edema	17	42	9	16	29	0

Table 3 Percent organ weights relative to live body weights and standard deviations for Taricha granulosa in Experiment 2. Total dosage to experimentals was 650 rads. Controls received 0 rads. N = 8 individuals sacrificed except where an asterisk indicates 7.

	Spleen	Liver	Kidney
Day 1			
Single	0.51 ± 0.15	6.4 ± 1.9	0.73 ± 0.15
Two fraction	0.49 ± 0.19	6.6 ± 1.9	0.82 ± 0.15
Control	0.40 ± 0.15	7.5 ± 2.0	0.77 ± 0.08
Day 3			
Single	0.42 ± 0.09	5.9 ± 1.4	0.73 ± 0.12
Two fraction	0.32 ± 0.12	6.2 ± 1.4	0.79 ± 0.10
Control	0.37 ± 0.08	6.5 ± 1.4	0.83 ± 0.07
Day 7			
Single	0.34 ± 0.14	5.0 ± 1.6	0.65 ± 0.24
Two fraction	0.26 ± 0.10	5.5 ± 0.71	0.81 ± 0.11
Control	0.53 ± 0.29	6.8 ± 0.77	0.82 ± 0.07
Day 9			
Single	0.35 ± 0.14	5.6 ± 2.1	0.76 ± 0.10
Two fraction	0.41 ± 0.18	5.6 ± 1.2	0.71 ± 0.25
Control	0.39 ± 0.16	7.0 ± 2.3	0.89 ± 0.15
Day 20			
Single	0.27 ± 0.18	4.0 ± 0.73	0.77 ± 0.12
Two fraction	0.27 ± 0.09	5.7 ± 1.3	0.84 ± 0.08
Control	0.29 ± 0.07	6.9 ± 1.6	0.78 ± 0.11
Day 45			
Single*	0.17 ± 0.05	4.8 ± 1.5	0.85 ± 0.16
Two fraction*	0.26 ± 0.14	4.4 ± 1.5	0.84 ± 0.11
Control	0.28 ± 0.17	7.2 ± 1.8	0.85 ± 0.05