to the donor fibroblasts. The data also show the reliability of using normal serum as a reference standard for killing in an isologous system, in contrast to the significant variation shown by normal serum when used as a standard in the analysis of heterologous cell lines (*8*). The data presented in Table 2 represent cells that survived treatment and were attached to plates 24 hours after seeding. To determine whether immune serum had any effect on the growth of surviving attached cells, sample plates from each reaction mixture were incubated for 3 and 6 days and stained, and the attached cells were then enumerated. The cells that survived killing by immune serums grew at the same rate as did those treated with normal and heated serums, and the percentage of cells killed, calculated from data at days 3 and 6, closely paralleled that at day 1. The variability evident in the immune serums and the fact that only four of six animals gave detectable response suggest that the antibody response was not the maximum obtainable. In addition, even with immune serums which could kill (third series serums), we could not detect antibody by complement fixation with donor-cell suspensions as antigen.

There are several possible interpretations for these observations. We may be dealing with genetic differences governing relatively few antigens, and these particular antigens may be only weakly antigenic. Another possibility is that cultured fibroblasts may be deficient in isoantigen molecules, or that their spatial arrangement prevents maximum exposure during immunization or killing experiments. We have not determined whether hyperimmunization will enhance the killing capacity of the antiserum, or the optimum concentration of complement necessary for maximum killing.

We conclude that the demonstration of killing of freshly cultured cells by isoimmune serums offers the possibility of utilizing antigenic markers in genetic analysis of these cell strains (*9*).

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X-Rays: Are There Cyclic Variations in Radiosensitivity?

Abstract. *When adult CF1 mice of either sex were x-irradiated at various times of day and under various conditions of light and dark, their responses showed no cyclic variations. Radiodiagnosis and radiotherapy may be practiced with equal safety at all times.*

Pizzarello *et al.* (*1*) recently reported that anesthetized rats, when treated with x-irradiation (to 900 r) at night, all died within 13 days, while other rats treated similarly in the morning all survived for more than 130 days. Untreated controls, irradiated at the same two times of day, also responded quite differently. Thus it was suggested that the biological responses of animals at 9 A.M. and at 9 P.M. are different, and that exposures at night are the more deleterious. This contention is of such importance to radiology that we have investigated the matter on a large scale, with variations in time of day as well as in the light-dark cycle (*2*).

A total of 2347 mice of two strains (CF1 and ICR) and both sexes were given x-irradiation under carefully controlled conditions, with and without prior anesthetization, at levels of exposure near the LD/50/30 range, in the morning and in the evening, to determine whether there might be any variations in radiosensitivity, as reported previously (*1*). There was no statistical evidence that x-ray treatment in the evening is more deleterious than the same treatment in the morning.

Mice irradiated at 6 A.M. did not survive quite as well as those exposed at 12 noon. This is explained on the basis of the metabolic activity associated with the nocturnal habits of these animals, which eat and mate at night and sleep during the day. There was no difference in the survival between animals irradiated in the morning and animals irradiated in the evening, or in animals irradiated before noon and animals irradiated before midnight.

The anesthetic sodium pentobarbital (Nembutal) afforded no protection to either males or females irradiated in the morning or in the evening, when they were exposed to supralethal doses of x-rays. There was high and rapid mortality at all times.

Since the biological variables are usually greater and more numerous than the physical variables in most experiments with x-irradiation, it seems obvious that all intrinsic and extrinsic variables must be eliminated or controlled and that large groups of animals are required to achieve statistically significant results involving lethality. With all variables except time of day properly controlled, there seems to be no evidence that mice are more radiosensitive in the evening than they are in the morning. In fact, to the contrary, our studies suggest that there are no cyclic variations in radiosensitivity—at least for mice.

Sexually mature males and females of the CF1 and ICR strains of mice were used. With the exception of one group of 14-month-old ex-breeder females, all were 3 to 4 months old.

The mice were kept eight in a mouse box (series 4 had six per box) under standard laboratory conditions of temperature, humidity, and daily fluctuations of light and dark, except where otherwise noted. After x-irradiation, daily checks of deaths were made.

The physical factors of x-irradiation were as follows: Parallel x-ray tubes, arranged so that the mice were exposed to a cross-fire, were run at 184 kv(peak), 30 ma, with 0.28-mm Cu and 0.50-mm Al filters having an aggregate half-value layer of 0.6 mm of Cu. In the first series the tubes were set at a target distance of 30 cm, with an air dose rate of 300 r/min, and in the later series the target distance was 28.5 cm, so that the air dose was 325 r/min. Depending upon the dose to be delivered, the exposures ranged from 2 minutes to 2 minutes and 10 seconds for the unanesthetized mice, and up to 3 minutes (975 r) for the anesthetized mice. All exposures were therefore acute. During exposure the mice were confined, five at a time, in a plastic box 13 cm in diameter and 4 cm high (inside measurements). The cover and sides were porous. The calculated dose in air approximated very closely the delivered dose to each mouse because the absorption by the plastic container

was balanced by the scatter from the bodies of the mice. The output of the x-ray tubes was calibrated with a Victoreen 250 r meter before and after irradiation both morning and evening. The output, under identical conditions, varied less than 2 percent and in most cases was much less.

Only the anesthetized mice were immobilized. The anesthetic was similar to that used in the prior series (*1*), namely sodium pentobarbital (Nembutal), given intraperitoneally. Since the mice were all close to 30 g in weight, a uniform dose of 0.3 ml of a 10 percent solution in saline was given. This dose completely anesthetized the mice within a few minutes, and the effect lasted more than 1 hour. When anesthetized mice were irradiated they were distributed evenly within the irradiation box to insure equal exposure.

The variables included time of day, shifting of the light and dark cycles, and radiation dose. The lethality data from 2347 mice are included in this report.

In the first series, involving 395 mice, the x-irradiations took place between 8:35 and 10:25 A.M. and between 8:25 and 9:30 P.M. Although males are more radiosensitive than females, they were given 650 r and the females 600 r. Because of possible diurnal effects in radiosensitivity, it was felt that for the less sensitive females a 600-r exposure would reveal any enhanced sensitivity, while for the more radiosensitive males a 650-r exposure might reveal any reduced sensitivity associated with the exposures in the morning and evening. Of the unanesthetized mice, 40 percent of the females irradiated at night survived the 30-day test period, while 35 percent of those exposed in the morning survived. Males irradiated at night showed 9.6-percent survival, those irradiated in the morning 2.1 percent. While the disparities are not statistically significant, in each case there was slightly better survival when the irradiation occurred in the evening.

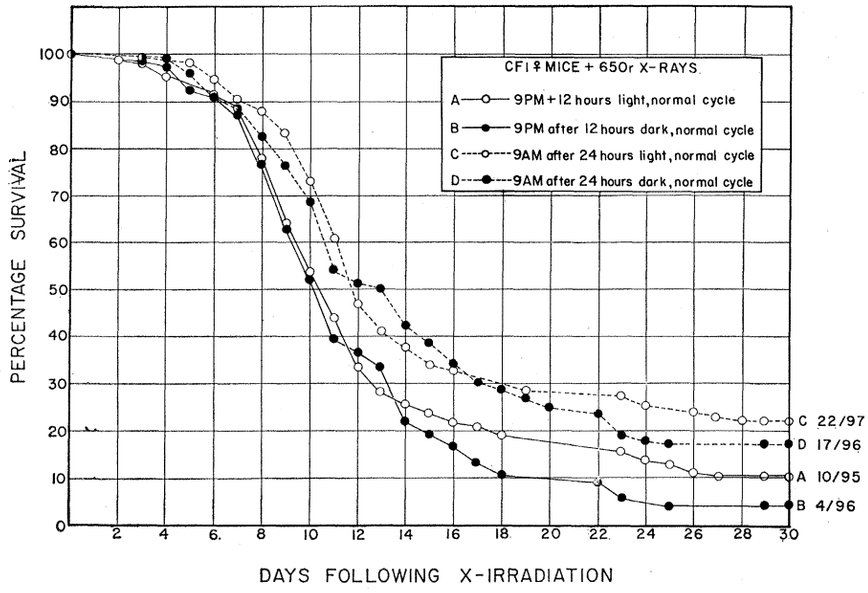

Fig. 1. Survival of mice x-rayed at 9 A.M. and 9 P.M.

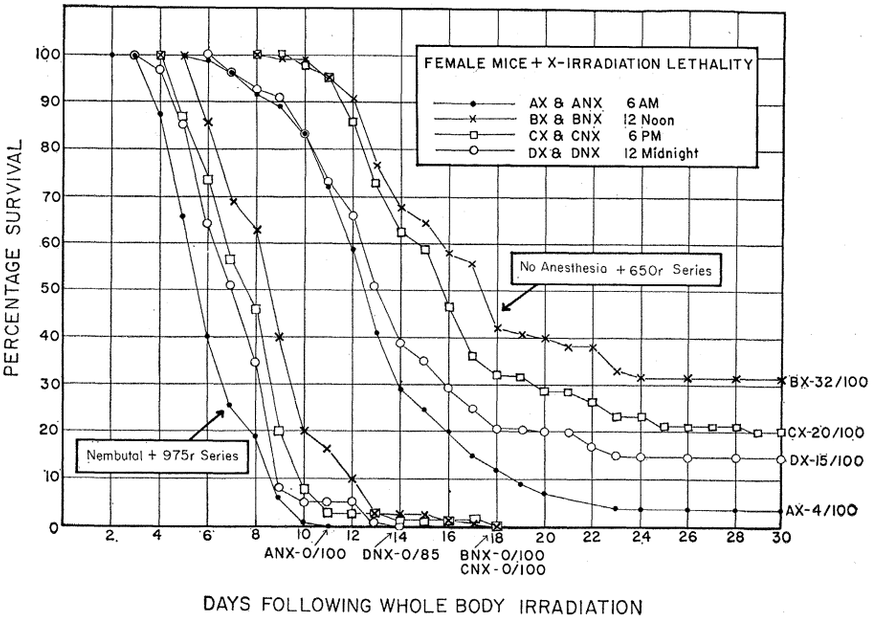

Fig. 2. Survival of mice x-rayed at 6-hour intervals beginning at 6 A.M.

Simultaneously another group of similar mice were anesthetized, and then irradiated as in the first series. Here again the statistically insignificant differences showed slightly better survival of both males and females irradiated in the evening.

In a third series involving 384 mice which were all ex-breeder females of the same age (14 months), the night-day, dark-light cycles were so altered that some were kept in light for 12 hours, irradiated, and continued in light for 24 hours more; others were kept in total darkness for 12 hours, irradiated, and returned to total darkness for another 12 hours; still others were kept in light for 24 hours, irradiated, and continued in light for another 12 hours; and the final group was kept in darkness for 24 hours, irradiated, and returned to the normal day-night cycle. Thus, there was not only a doubling of normal light and dark phases before and after x-irradiation, but the time of day and the lighting conditions were also thrown out of phase.

The data on survival for this series (Fig. 1) indicate that none of these variables altered the results significantly (*3*). When the data are subjected to statistical analysis there is a suggestion that the four curves do indicate factors which may slightly affect survival because, when curves *B* and *C* are compared, there appears to be some significance ($\chi^2 = 14.7$, when 7.82 is significant). The mice kept in total darkness for 24 hours and irradiated at 9 P.M. showed slightly less sur-

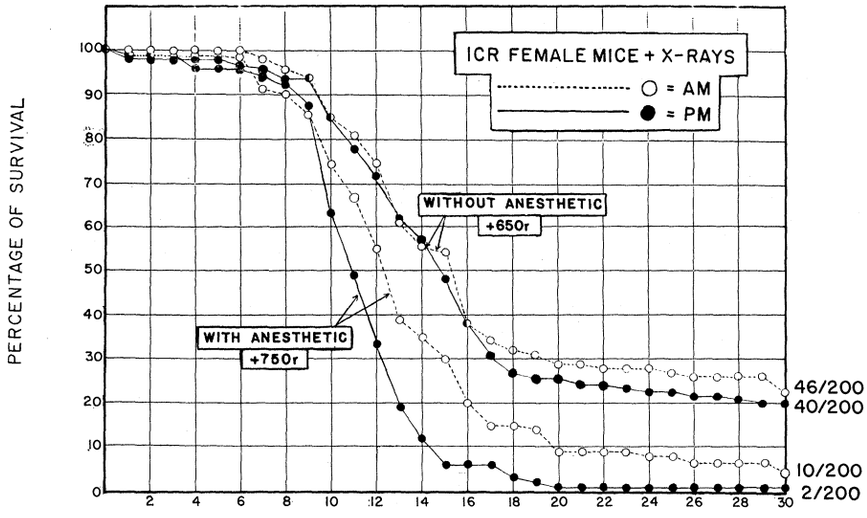

Fig. 3. Survival of anesthetized and x-rayed mice.

vival than mice kept in light for 24 hours and irradiated at 9 A.M. Since both light and time of day were involved, the data do not indicate which of the two variables might be responsible or whether there is evidence of synergism. It should be pointed out that mice are nocturnal animals, and the extension of the dark phase would extend their period of activity, thus increasing their radiosensitivity. When one compares the four curves and the final survival percentages, there is no evidence of any significance, since the two extremes balance out to equal the intermediates.

In the next series, involving 785 mice, irradiations occurred at 6-hour intervals throughout the 24-hour period, under normal light-dark, day-night conditions. The times were 6 A.M., noon, 6 P.M., and midnight. Four hundred mice were exposed without prior anesthetization (100 each time), and 385 were exposed after anesthetization. The data are given in Fig. 2.

Among unanesthetized mice, irradiation at 6 A.M. gave slightly poorer survival than irradiation at noon ($\chi^2 = 26.57$ over significance of 7.82). However, when the combined data from 6 A.M. and noon are compared with the combined data from 6 P.M. and midnight, there is no significance whatever. This suggests that irradiation from 6 A.M. until noon has the same effect as irradiation from 6 P.M. until midnight. When the four curves are analyzed together and separately, there is slight evidence that either the light or time variables affect survival. The poorest survival occurred when the mice were exposed at 6 A.M., the best when the mice were exposed at noon. The difference does not compare with that reported by Pizzarello *et al.* (*1*).

In the final series (800 ICR female mice), the original variables of 9 A.M. and 9 P.M. were used, and a lower dose of x-rays was given to the anesthetized mice (750 r). Of the mice irradiated in the morning, 23 percent survived for 30 days. Of those irradiated in the evening, 20 percent survived. Likewise, the anesthetized mice showed survival values of 5 and 1 percent (Fig. 3). Thus, with these larger groups of similar mice, it is evident that there is absolutely no statistically significant difference in the survival of mice irradiated in the morning or evening.

Radiotherapy has been practiced around the clock for many years without fear, suspicion, or evidence that the biological system would react differently to ionizing radiation at different times of the day. The recent study of Pizzarello *et al.* (*1*) led these workers to state that "radiosensitivity may vary as a function of irradiation in the light phase or the dark phase of a 24-hour day."

Under the supervision of a radiophysicist and with the checks employed by every trained radiotherapist, it is very unlikely that major fluctuations which might be related to the time of day and which would go unnoticed could occur in the output of x-ray machines. However, there are many intrinsic variables which affect the radiosensitivity of any living organism (*4–6*). When large numbers of apparently similar animals are used, minor variations tend to be balanced out in the controls and experimentals. The major objection to the thesis of Pizzarello *et al.* (*1*) is that the conclusions were based upon inadequate statistics.

Diurnal fluctuations occur in many animal activities. Hence the concept of fluctuations in radiosensitivity is indeed plausible. Rodents are nocturnal animals, doing their eating and mating at night for the most part. They are generally quite inactive during the day, particularly around mid-day. The mice which showed a slightly higher mortality at 6 A.M. than those irradiated at noon were probably slightly more radiosensitive because of a night of activity, while those irradiated at noon were awakened from the depression of sleep. This explanation is further supported by the data of Fig. 1 wherein the lengthened dark period was correlated with the slightly greater radiosensitivity. Exercise, occurring at night for these nocturnal animals, has long been recognized as enhancing the deleterious effects of whole body x-irradiation (*5*).

Data on lethality for mice are reproducible, so that fluctuations of as little as 10 r can be detected (*7*), provided that all other variables are balanced out. In our experiments the extrinsic variables were controlled by having different individuals solely responsible for the various aspects of the experiments. The sole variables were the light and the time cycles, which were strictly controlled in our investigations.

Some anesthetics and narcotics will affect the LD/50/30 values (*8*). Generally they are not radioprotective, and those that are cannot compare in effectiveness with certain other substances. However, since Nembutal had been used in the prior series, we decided to use the same anesthetic to determine whether it was truly an effective agent. In every instance prior anesthetization with Nembutal augmented the expected lethality of the x-rays.

Finally, it must be pointed out that the earlier study was based upon rats, whereas our study is based entirely upon mice. Extrapolations are always unwise, even between closely related species, and data from rats cannot be refuted by data from mice. However, the data of our study, in which some 2347 mice were used under various controlled conditions of light and time, do not support the contentions of Pizzarello *et al.* (*1*) that there are cyclic variations in radiosensitivity, specifically, that irradiations at night are more dangerous than those in the morning. If Pizzarello *et al.*

could substantiate their contentions with adequate statistics, all radiologists would have to be alerted to the possibility that the human body might be differentially affected by irradiation at different times of the day. Until that time, radiotherapy can be practiced with the usual safety around the clock.

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18 July 1963

Crystallization of SE Polyoma Virus

Abtract. *Crystals have been obtained from purified preparations of SE polyoma virus. Specific infectivity measurements of the mother liquor, wash fractions, and the dissolved crystals indicate the viral nature of the crystals.*

Since the initial crystallization of poliovirus by Schaffer and Schwerdt (*1*), several animal viruses containing ribonucleic acid have been crystallized (*2*). It is the purpose of this communication to describe the crystallization of SE (Stuart and Eddy) polyoma, an animal virus that contains deoxyribonucleic acid (DNA) (*3*). This is believed to be the first report of the crystallization of a DNA animal virus. The crystallization of *Tipula iridescent*, an insect virus that contains DNA, was reported by Williams and Smith (*4*).

SE polyoma [Eddy strain 4B5 (*5*)] was grown either in tissue cultures of the mouse embryo or in cultures of kidneys of mice inoculated with the virus a few days after birth, according to the procedure of Winocour (*6*).

The cultures were harvested when most of the cells had lysed. The cells were scraped with a rubber spatula and the cells and medium aspirated into a collection flask. The plates were washed with one ml of saline-borate buffer, *p*H 9 (9 parts of 0.15*M* NaCl to 1 part of saturated sodium tetraborate), and the washings collected with the cells and medium. The alkaline cell suspension was centrifuged at 1500*g* for 20 minutes at 4°C. The virus particles contained in both the 1500*g* supernatant and the 1500*g* packed cell fractions were purified by the procedure described here (*7*).

The 1500*g* packed cell pellet was extracted with saline-borate buffer by homogenization with an all glass homogenizer. The homogenate was centrifuged in the Spinco No. 30 rotor at 10,000 rev/min for 30 minutes. The pellet was discarded. Enough 5 percent sodium deoxycholate-0.01*M* tris, *p*H 9, was added to the supernatant fraction so that a final concentration of 0.5 percent was attained and the solution was swirled at room temperature for 15 minutes. The virus in this fraction was concentrated by centrifugation in the No. 30 rotor at 30,000 rev/min for 2 to 3 hours. The pellet obtained at high speed was suspended in 0.5 percent sodium deoxycholate-0.01*M* tris, *p*H 9, by homogenization, and the differential centrifugation cycle was repeated. The pellet finally obtained at the high speed was homogenized in 5 ml of unbuffered saline and centrifuged in the Spinco SW 39 rotor at 10,000 rev/min for 30 minutes. The virus in the supernatant fraction was obtained as a clear gel-like pellet by centrifugation in the SW 39 rotor at 36,000 rev/min for 2 hours.

The virus in the 1500*g* supernatant fraction was concentrated by centrifugation in the No. 30 rotor at 30,000 rev/min for 2 to 3 hours. The pellets were pooled and suspended in 0.5 percent sodium deoxycholate-0.01*M* tris, *p*H 9, by homogenization. The virus in this fraction was then also subjected to the same differential centrifugation procedure.

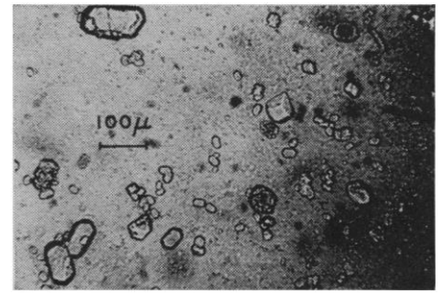

Fig. 1. Crystals of SE polyoma virus. Visible light.

Crystals were seen to form almost immediately on the addition of a few drops of water to a pellet obtained in the SW 39 rotor; this pellet being the final product resulting from purification of polyoma virus, derived initially from the 1500*g* packed cell fraction. On standing at 4°C for several days, the entire pellet had turned crystalline. The crystals appear to have the form of rhombic dodecahedra (Fig. 1).

Evidence that the crystals were com-

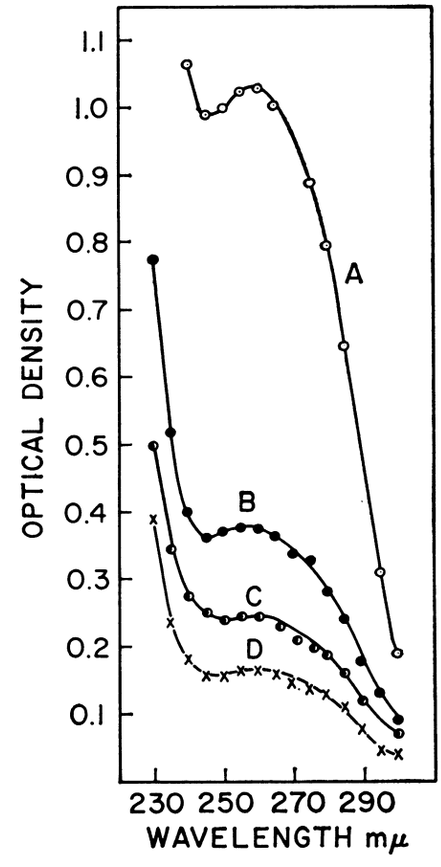

Fig. 2. Ultraviolet absorption spectra of wash fractions (Table 1) measured with the Zeiss spectrophotometer. *A*, mother liquor; *B*, wash No. 1; *C*, crystalline virus; *D*, wash No. 2.