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The Effect of Dose Rate on the Survival of S3 HeLa Cells Exposed to Cobalt 60 Gamma Radiation

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THE EFFECT OF DOSE RATE ON THE SURVIVAL OF S3 HELA CELLS EXPOSED TO COBALT 60 GAMMA RADIATION

by

Joel Sanders Bedford

B.A., University of Colorado, 1961

A Thesis submitted to the Faculty of the Graduate School of the University of Colorado in partial fulfillment of the requirements for the Degree Master of Science Department of Radiology

1963
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A most sincere appreciation is expressed to Dr. Eric J. Hall for his direction and unfailing patience throughout the investigation and also for his criticism during the writing of this thesis.

This Thesis for the M.S. degree by Joel Sanders Bedford has been approved for the Department of Radiology by

An especial thanks, also, to Mr. Charles Waldren for many helpful suggestions, to Dorothy Inselman for her painstaking work on the manuscript and to my parents and wife for their loving encouragement.

Date Aug 12th 1963.
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A most sincere appreciation is expressed to Dr. Eric J. Hall for his direction and unfailing patience throughout the investigation and also for his criticism during the writing of this thesis.

The author is indebted to Dr. Glen V. Dalrymple for his guidance in the statistical and mathematical evaluation of the experimental results; his help is gratefully acknowledged.

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An especial thanks, also, to Mr. Charles Waldren for many helpful suggestions, to Dorothy Inselman for her painstaking typing of the final manuscript and to my parents and wife for their help and encouragement.

ABSTRACT

The investigation was designed to determine whether a dose of radiation delivered to a population of single cells grown in vitro, using the techniques originally described to a population of single cells and the fraction of the population "reproductively surviving," i.e. able to reproduce indefinitely.

Doses of 100 to 1000 rads were delivered at dose-rates of 2.37, 16.9, and 44.9 rads per minute. At every dose level, 18 plates containing pre-irradiated feeder layers were inoculated with an equal number of 83 HeLa cells and divided into three sets of 6 plates. All three sets received
ABSTRACT

Bedford, Joel Sanders (M.S., Radiobiology)

The Effect of Dose Rate on the Survival of S3 HeLa Cells Exposed to Cobalt 60 Gamma Radiation

Thesis directed by:

Visiting Assistant Professor Eric J. Hall

The investigation was designed to determine whether the rate at which a dose of ionizing radiation is delivered affects its ability to sterilize single cells grown in vitro.

The cells were cultured using the techniques originally described by Puck and his co-workers, whereby mammalian cells may be treated as independent microorganisms. This technique enables the description of a quantitative relationship between the dose delivered to a population of single cells and the fraction of the population "reproductively surviving," i.e. able to reproduce indefinitely.

Doses of 100 to 1000 rads were delivered at dose-rates of 2.37, 16.9, and 44.9 rads per minute. At every dose level, 18 plates containing pre-irradiated feeder layers were inoculated with an equal number of S3 HeLa cells and divided into three sets of 6 plates. All three sets received
the same total dose of cobalt 60 gamma radiation, but the first set received its dose at a rate of 2.37 rads per minute, the second, at 16.9 rads per minute, and the third at 44.9 rads per minute. These plates were then incubated for a period of about two weeks, after which they were fixed, stained and the number of colonies on each plate counted.

In every case, it was found that the effectiveness of the radiation in sterilizing the cells was increased when the dose-rate was increased. The magnitude of the dose-rate effect at the cellular level was sufficient to account for published reports of variation in LD 50/30 for whole-body irradiated small mammals.

This abstract of about 254 words is approved as to form and content. I recommend its publication.

Signed

Instructor in Charge of Dissertation
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INTRODUCTION

All living organisms on earth are exposed, in greater or lesser degrees, to ionizing radiations. These radiations may be of cosmic origins, arise from the decay of naturally occurring unstable atomic nuclei in the earth itself, or be emitted from man-made isotopes and high voltage electrical machines. However diverse their origin, they produce essentially the same biological effects.

The Nature and Absorption of Ionizing Radiations.

Ionizing radiations have been classified into two major categories; electromagnetic and corpuscular.

Electromagnetic radiations may be described as the propagation of energy associated with electric and magnetic fields, varying in mutually perpendicular planes where the direction of propagation is along the line common to both planes. The velocity in vacuo is always constant, at approximately $3 \times 10^{10}$ centimeters per second; and is equal to the product of the wavelength and the frequency of the radiation.

In order to explain a variety of observations concerning the properties of electromagnetic radiations, it becomes necessary to describe them as having characteristics of both wave motion and the motion of photons or discrete packets of energy. The
energy of the photon ($E$), is equal to the product of its frequency ($\nu$) and Planck's constant ($h$). This dual nature may be difficult to accept if one finds it necessary to resolve all of the observations of a phenomenon into a single unifying concept. However, the only way of defining a particle or a wave is to measure all its properties simultaneously, and Heisenberg's Uncertainty Principle tells us that there is a limit to the accuracy with which we may determine the position and momentum of a particle simultaneously. As measurements of momentum are increasingly refined, the act of measurement itself increases the error in determining position; conversely, more accurate observations of position lead to an uncertainty in momentum. Thus, all we are able to say is that particles and waves are complementary descriptions of the same phenomenon.(1)

The so-called corpuscular radiations differ in that they consist of a stream of particles. The particles may carry a positive or negative charge or no charge at all; masses of the particles differ by a factor of about $10^4$. Common examples of these types of radiations are alpha particles protons and deuterons, which are heavy positively charged particles; electrons, which are light negatively charged particles, and neutrons which are relatively massive...
but carry no electric charge.

In order to produce ionization, the radiation, whether electromagnetic or corpuscular, must possess sufficient energy to eject an electron from an atom or molecule of the material in which it is absorbed. Electromagnetic radiation with a wavelength less than about 100 Ångstrom units, corresponding to photon energies greater than 124 electron volts, is generally considered to be ionizing, as are corpuscular radiations with kinetic energies greater than approximately 100 electron volts.¹

In this investigation, we are concerned primarily with the action of ionizing electromagnetic radiation, particularly gamma-rays. X- and gamma-rays are essentially identical in nature, the only difference being the mode of production. X-rays are produced as a result of electrons losing energy. This may result from: (1) the rapid deceleration of a fast moving electron due to strong forces acting upon it when it passes near an atomic nucleus ("bremstrahlung" or "braking radiation"), or (2) changes in electronic energy states within

¹ The value of 100 electron volts was obtained from data on the variation of the mean energy per primary ionization in water with the energy of the incident particle. This value appears to be, at present, the best approximation for particles whose energy is of the order of 1 to 10 Mev, although it may be as low as 70 electron volts per primary ionization for very low energy electrons. (2)
the atom whereby an electron loses potential energy. Provided the energy loss is great enough, a photon will be emitted with a wavelength in the X-ray range. In contrast, gamma-rays originate as a result of energy transitions within the atomic nucleus. If an unstable atomic nucleus is decaying to a stable state, there may be several transition states accompanied by the emission of $\beta$ or $\alpha$ particles. Consequently, a transition of long wavelength produce electron oscillation. The ions in biological systems are another example; these are made to oscillate by certain long wavelength radiations producing localized heating effects. The somewhat shorter wavelength radiations of the infrared region may cause the atom of a dipolar molecule to vibrate which can result in large streages and perhaps even rupture chemical bonds. Proceeding in wavelength through the visible region of light where the energy is great enough to raise outer electrons of atoms to deviated from their original path without loss of energy, while the remainder are truly absorbed and their energy used to ionize or excite the atoms and molecules of the absorbing medium. The absorption of energy results from an interaction between the photons of electromagnetic radiation and the electrons of an absorber. It is found that the more nearly matched the energy of the photon and the binding energy of the electron, the higher the probability of the radiation transferring its energy to that particle. Thus, the probability of absorption is maximal when the energy of the bound electron and of the
radiation is equal; conversely, for each energy state of an electron there is a characteristic wavelength of radiation that it maximally absorbs.

Completely free ions or charges with no inertia may absorb any wavelength of radiation. This condition is approached in the metal conductors where even radio waves of long wavelength produce electron oscillation. The ions in biological systems are another example; these are made to oscillate by certain long wavelength radiations producing localized heating effects. The somewhat shorter wavelength radiations of the infrared region may cause the atoms of a dipolar molecule to vibrate which can result in large stresses and perhaps even rupture chemical bonds. Proceeding in wavelength through the visible region of light where the energy is great enough to raise outer electrons of atoms to higher energy levels and through the even shorter wavelength region of ultraviolet light where not only outer, but also, inner electrons may be excited by the radiant energy, we arrive at what is sometimes called the "transition" region. The radiations here have a range of wavelengths between approximately 1000Å and 10Å and are so readily absorbed in air and biological materials that their effects can only be studied on very small objects in a vacuum, and thus, little is known of their action on living matter. As we further
proceed downward in the electromagnetic spectrum of wave-lengths, we find that the probability of absorption correspondingly diminishes but always remains finite and when absorption does occur, the result is almost invariably the ionization of an atom or molecule.

As mentioned earlier, high energy electromagnetic radiation produces its ionization when it results in the emission of a charged particle as it is absorbed in an atom. This secondary charged particle may then produce further ionization when it transfers some of its energy to the orbital electrons of other atoms. In each case, the amount of energy transferred upon collision is a function of the momentum and charge of the particle and also the conditions under which the impact occurs. After a collision, the particle will continue the process until it has lost all or most of its energy.

There are three main mechanisms by which electro-magnetic radiation may transfer its energy to a bound electron, namely, the photoelectric effect, the Compton effect, and pair production.

In the photoelectric effect the entire energy of the photon is transferred to an electron of the absorbing atom which is then ejected from that atom. Part of the photon energy is spent in removing the electron from the atom and
the remainder appears as kinetic energy of the freed electron. If the electromagnetic radiation does not have enough energy to remove an electron, excitation rather than ionization will occur. Photoelectric absorption occurs predominately when the absorber is of high atomic number and the radiation energy is relatively low.

The Compton effect involves only a certain fraction of the photon energy being lost when it interacts with a loosely bound electron. The photon recoils at an angle between 0 and 180 degrees from its original path and the electron is dislodged from the atom with an energy equal to the energy lost by the photon minus whatever binding energy it had in association with the atom. The recoil photon may then continue a series of similar encounters, each time losing more energy until it experiences a photoelectric absorption. The Compton effect occurs over a wide range of photon energies, but is predominate at energies between about 100 Kev and 1 Mev, where its relative incidence is not overshadowed by photoelectric absorption or pair production.

Pair production occurs when a photon, whose energy is greater than 1.02 Mev, approaches the powerful fields close

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1 Kev = one thousand electron volts; 1 Mev = one million electron volts.
to an atomic nucleus where its energy is converted into a positron-electron pair. Of the total photon energy, 1.02 Mev is spent in producing the rest masses of the pair, and any energy the photon had in excess of this is shared equally between the two particles in the form of kinetic energy. Subsequently, both particles will ionize along separate paths in the usual manner, but the positron, after it has lost most of its energy, is annihilated as it approaches an electron and the rest mass of the two particles is radiated as a pair of 0.511 Mev photons.

As a charged particle travels through a given medium, by local energy releases, it will leave in its path a number of atoms or molecules that have been excited or ionized. The distribution of these local energy releases has been described in terms of specific ionization defined to be the ion density expressed as ion pairs per unit of path length, or linear energy transfer (LET) expressed as the energy loss of the particle in electron volts per unit of path length. The greater the charge of the particle and the smaller its velocity, the greater is its linear energy transfer and specific ionization. Complications arise with both of these descriptions, however, since as the particle slows down when it passes through matter, there is a continuous increase in LET and specific ionization. As a compromise, average LET
or average specific ionization are terms commonly used even though the usefulness of this concept may itself be in question. In any case, since a beam of protons or of α particles will have a limited range unless their energy is very great, beams with a greater penetrating power such as X- or gamma-rays are more suitable for most radiobiological experiments. Charged particles, set in motion by high energy electromagnetic radiation, will have a wide variety of energies depending on the processes of absorption and ionization resulting in their production; their energy therefore will likewise be absorbed over a range of values of linear energy transfer. Some numerical values quoted from the second edition of The Physics of Radiology will serve as an indication of this range. For cobalt 60, energy is lost by tracks with values of LET from about 0.2 to 2.0 Kev per micron. For 250 Kev x-rays, the range is from about 0.4 to 40 Kev per micron with a relative maximum at 1.4 Kev per micron. Neutrons set protons into motion in the process of slowing down, and the energy loss is then over a range from 8 to 90 Kev per micron. With 5.5 Mev α particles, the range of LET values is from about 90 to 270 Kev per micron. (2)

Dosimetry

Radiation can only be detected and measured as a result
of events occurring when it interacts with matter. To measure ionizing radiation, the obvious thing to do is to measure the amount of ionization produced. In solids or liquids this is very difficult, but in gases it may be accomplished simply and accurately. Because of its availability, air has been most widely used as the gas, and ionization produced in it by the passage of ionizing radiation may be determined with what is known as a "free-air" ionization chamber. In this instrument the ions produced in a known volume of air are collected by a pair of oppositely charged plates and the total charge collected is measured with an electrometer. Prior to 1928 the quantity of radiation delivered was determined by a variety of techniques, and a unit of radiation dose was not clearly defined. In 1925 at the first International Congress of Radiology, the International Commission on Radiological Units (I.C.R.U.) was formed. At this time, the need for a defined unit of radiation dose was discussed. At the second meeting in 1928 the roentgen unit was defined and formally adopted, and although it has since undergone several revisions, the concept has remained essentially unchanged. The latest definition of the roentgen for photon energies of less than 3 Mev was adopted by the International Commission on Radiological Units in 1956, and is quoted in the National Bureau of
Standards Handbook 62 as follows:

"Exposure dose of X- or gamma-radiation at a certain place is a measure of the radiation that is based upon its ability to produce ionization.

The unit of exposure dose of X- or gamma-radiation is the roentgen (r).

One roentgen is an exposure dose of X- or gamma-radiation such that the associated corpuscular emission per 0.001293 grams of air, produces in air, ions carrying one electrostatic unit of quantity of electricity of either sign." (3)

The "free-air" ionization chamber mentioned above is generally used as a primary standard with which other instruments may be calibrated by comparison.

For routine dose measurements, a pre-calibrated thimble chamber or "Bragg-Gray cavity" is used. The wall of the chamber is made of material which is "air equivalent" i.e., has an effective atomic number close to that of air. The wall thickness must be greater than the maximum range of the secondary electrons in order to achieve electron equilibrium.

In the United States the most commonly used dosimeter is the Victoreen condenser r-meter. In this instrument the chamber with its built in condenser is charged to a predetermined voltage and exposed to the dose of radiation being measured. The voltage across the condenser in the chamber is then remeasured; the dose to which the chamber was exposed is proportional to the difference in initial and final voltage readings. If the air in the chamber was not at Standard
Temperature and Pressure when the dose was measured, and also, since the response of the chamber may vary slightly with different photon energies, corrections must be applied to obtain the final dose in roentgens. It should be noted that the thimble chamber actually measures ionization in air, the quantity to which the roentgen refers. However, when studying biological effects, the important consideration is not the ionization produced by the radiation in air, but the energy absorbed in the biological material. In 1956, therefore, the International Commission on Radiological Units recommended the adoption of a unit of radiation absorbed dose. They defined this unit, the rad, as the absorption of 100 ergs per gram in any medium at the point of interest.

The exposure dose at a particular point of interest, measured in roentgens, can be used to calculate the absorbed dose in rads for X- and gamma-radiation, using the theory developed independently by W.H. Bragg and L.H. Gray. (4) The dose absorbed (in rads) by the medium at the point of interest, is equal to the product of the exposure dose in roentgens and a proportionality factor \( f \). This roentgen to rad conversion factor is defined as the product of the absorbed dose in air per roentgen of exposure, and the ratio of the energy absorption coefficient of the medium to the energy absorption coefficient of air. Photon energy and
atomic number of the absorbing material are factors that affect the value of "f". Some numerical examples of the "f" factor are shown in Table I for photon energies of 0.01 Mev, 0.1 Mev, and 1.0 Mev in water, bone, and muscle. (5)

TABLE I

<table>
<thead>
<tr>
<th>Photon energy</th>
<th>Water</th>
<th>Bone</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 Mev</td>
<td>0.920</td>
<td>3.58</td>
<td>0.933</td>
</tr>
<tr>
<td>0.1 Mev</td>
<td>0.957</td>
<td>1.47</td>
<td>0.957</td>
</tr>
<tr>
<td>1.0 Mev</td>
<td>0.974</td>
<td>0.927</td>
<td>0.965</td>
</tr>
</tbody>
</table>

Other methods of estimating absorbed dose include the measurement of: (a) changes in oxidation state of a chemical compound (discussed later in more detail), (b) the blackening of the emulsion on a photographic film, (c) fluorescence produced in certain types of glass, (d) changes in electrical resistance of some semi-conducting materials, and (e) the rise in temperature of the material in a calorimeter as a result of energy absorption and subsequent heat production.

When making dose measurements and evaluating the results of radiation experiments, it is of utmost importance that the difference in absorbed dose and exposure dose be realized.
Biological Effects of Ionizing Radiations

To obtain a feeling for what happens when ionizing radiation encounters living matter, let us consider briefly why ionizing radiation can be so destructive to a biological system.

One may justifiably ask why ions formed by radiation should lead to any particular harm in a biological organism since the organism functions, and indeed, depends upon countless ions in its normal state of metabolism. An answer may be found if we consider for a moment that the ions functional in living organisms exist in their most stable form. For example, the ions Na⁺ and Cl⁻ are much more stable than free sodium metal or chlorine gas. On the other hand, the negative ion produced by radiation is a free electron with kinetic energy that may be capable of disrupting other atoms or molecules while either the positive ion or free radical formed will not necessarily be a stable form, and if all its valencies are not satisfied it can be very reactive. The ions and free radicals which are formed will attack neighboring atoms and molecules randomly in attempting to regain a stable form and thereby disrupt the functional organization of the system.

Professor T.T. Puck, in a series of lectures on the action of ionizing radiation in living organisms, compared the results of irradiating, separately, a liter of water and
a liter of living matter and pointed out the following differences. When pure liquid water in its lowest possible energy state is subjected to a beam of ionizing radiation, a myriad of new ions and free radical hybrid species are formed, and when the irradiation is stopped all the species will react until the system has returned to its lowest possible energy state which is again, pure water. The energy originally absorbed is ultimately converted to heat. In contrast a biological system is not in its lowest possible energy state, and in fact, life only exists because of a delicate arrangement of molecules in a high energy state so that an orderly and continual "passing down" of energy from molecule to molecule may occur. In the case of water, after excitation or ionization the only state to which it can return is water, but with a complex biological molecule such as a protein or a nucleic acid there are many possible structures to which it may return after excitation or ionization and, in all probability, the structure will not be functional in the system. Thus, a knowledge of the nature of molecules in biological systems and the changes that occur in them when acted upon by ionizing radiation may at some time lead to an understanding of the mechanisms by which these forms of radiant energy lead to injury or death of an organism.
In studying radiation effects, barring moral and technical difficulties, the radiobiologist has as many biological tools as nature has provided plant and animal species. Much of the pioneering experimental research in radiobiology was done with such systems as bacteria, yeasts, fungi, *Arbacia* (marine animal of the Phylum Echinodermata), *Drosophila*, and *Vicia faba* (broad bean) roots; these systems are still widely used. The effect of ionizing radiation on whole mammalian organisms is certainly of great interest, and countless investigations have been carried out using mice, rats, guinea pigs, and other common small animals as experimental systems, as well as a few with larger species such as pigs, goats, dogs, and donkeys. Although the mechanism of radiation damage in whole mammals is exceedingly complex, involving not only cellular but integrated multicellular and systemic effects, many qualitative and quantitative end effects of irradiation treatment regimes have been clearly demonstrated. In varying degrees, the same is true of all organisms, from unicellular to multicellular. As Raymond E. Zirkle has stated, "...The beginning is the act of irradiation, and the end is the effect observed; there is considerable information about these and the prospects of getting more are good. The middle, frequently miscalled the 'latent period', is essentially a domain of ignorance wherein most of the
Culture methods which allow the formation of clones arising from a single cell have been a popular technique in quantitative microbiology for a number of years, and these methods have been fruitfully applied to radiobiological studies. Until 1956 these techniques were used only in connection with so-called "lower forms of life" (unicellular bacteria, yeasts, protozoa, etc.), and it was furthermore, a common opinion among biologists that mammalian cells could not be grown as individual units in vitro. However, in 1956, T.T. Puck and his co-workers demonstrated that this was not the case, and indeed, mammalian cells could, under suitable conditions, be treated as independent micro-organisms. This method of culture allows one to evaluate the effect of a particular agent on the organism. A known number of cells may be introduced into a petri dish containing suitable growth medium, and treated with the agent; after a period of incubation, the number of colonies formed in these dishes may be compared with the number of colonies in untreated control dishes.

An important point should here be recognized. When speaking of effects of an agent on a biological organism, any changes, whatsoever, in the integrity of the organism caused by the agent are implied. The radiation effect studied
here, using single cell culture techniques, is reproductive death, defined as the loss of the ability to proliferate indefinitely.

The Problem Stated.

The total energy absorption of ionizing radiation in ergs per gram (i.e. dose in rads) received by an organism is the most important single factor determining biological response. Also of importance, when the effect is believed to result from the passage of more than one ionizing particle, is the rate of energy absorption, (i.e. the dose-rate in rads per unit time). Reports in the literature as early as the nineteen twenties have described radiation effects on biological material which vary with the rate of energy absorption. These reports indicate that radiation dose-rate alters such effects as, division delay in chick tissue cultures, chromosome aberrations in *Trandescantia*, cleavage delay in *Arbacia* eggs, and growth inhibition of *Vicia faba* roots. (8, 9, 10, 11) More recently, especially in the past decade, a number of reports have appeared in the literature describing the effect of radiation dose-rate on the **L.D. 50/30** for whole body irradiated small mammals. (12 to 20) In every case, an increase

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1 The **L.D. 50/30** is defined as the dose, in rads, necessary to kill 50 per cent of a population of organisms within 30 days after irradiation.
in the L.D. 50/30 was observed when the dose-rate was decreased. It seemed of value to know whether this effect could be accounted for at the cellular level.

a) Maintenance of Stock Cultures. A stock of 83 HeLa cells was obtained from Dr. T. T. Puck of the Biophysics Department, University of Colorado Medical Center. The cells were routinely grown in loosely stoppered glass bottles in a medium consisting of 59 per cent Puck's Saline F, 26 per cent Puck's N16, and 15 per cent calf serum. The cellular environment was kept constant in a water-jacketed incubator maintained at 37.5 ± 0.2 degrees Centigrade, 90 to 100 per cent relative humidity, and was continuously flushed with a mixture of 5 per cent CO₂ in air so that the bicarbonate buffer system of the growth medium would be in equilibrium at a pH of approximately 7.2. Stock cultures were "farmed" and new bottles inoculated every three days. The culture, counting and plating techniques used in the Biophysics Department of the University of Colorado, described in Volume V of Methods in Enzymology (21) were closely followed and are here briefly summarized.

b) Experimental Procedure. On the day before an experi-

---

1 Calf Serum #4-0200 purchased from Flaw Laboratories Incorporated, 1710 Chapman Avenue, Rockvillle, Maryland.
MATERIALS AND METHODS USED

Culture of the Cells

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b) Experimental Procedure. On the day before an experiment a stock bottle in which the cells appeared healthy upon microscopic examination, and had multiplied to form a nearly confluent layer over the inside glass surface, a minimum of floating cells in the medium and the absence of macroscopic clumped areas of cells was considered desirable. Having selected a satisfactory stock culture, the cells were removed from the glass and dispersed for counting in the following manner:

The medium in the stock bottle was removed and the layer of attached cells washed twice with about 3 milliliters of a 0.05 per cent solution of 1:200 trypsin in Puck's Saline. The process was designed to remove any loosely attached or floating cells not removed with the medium. A further 3 milliliters of trypsin solution was added and the bottle placed in the incubator for about three minutes by which time most of the cells had become detached from the glass surface. The cell suspension was then gently pipetted into a complete growth medium which arrested the action of the trypsin. Hemocytometer were filled with a 0.1 milli-

---

1 Calf Serum #4-0200 purchased from Flow Laboratories Incorporated, 1710 Chapman Avenue, Rockville, Maryland.
ment a stock bottle was chosen in which the cells appeared healthy upon microscopic examination, and had multiplied to form a nearly confluent layer over the inside glass surface. A minimum of floating cells in the medium and the absence of macroscopic clumped areas of cells was considered desirable. Having selected a satisfactory stock culture, the cells were removed from the glass and dispersed for counting in the following manner.

The medium in the stock bottle was removed and the layer of attached cells washed twice with about 3 milliliters of an 0.05 per cent solution of 1:300 trypsin in Puck's Saline D1. The process was designed to remove any loosely attached or floating cells not removed with the medium. A further 3 milliliters of trypsin solution was added and the bottle placed in the incubator for about three minutes by which time most of the cells had become detached from the glass surface. The cell suspension was then gently pipetted to break up any clumps and the dispersed cells added to a sterile test tube containing about 0.5 milliliters of the complete growth medium which arrested the action of the trypsin. Using the suspension in the test tube, both chambers of an American Optical Hemocytometer were filled with a 0.1 milliliter pipette held horizontally during the transfer operation.
to avoid the cells settling toward the tip of the pipette. Counts were made of the number of cells in each chamber to obtain an estimate of the concentration of cells in the suspension; a total of about 400 cells were counted on each occasion. The desired concentration was obtained by serial dilutions with complete growth medium. 5 X 10^4 cells were then pipetted into each 60 millimeter Falconware plastic petri dish containing 5 milliliters of growth medium which had previously been placed in the incubator long enough for the medium to reach equilibrium temperature and pH. Care was taken to insure a uniform dispersion of cells in the suspension by gentle pipetting before inoculation. Each petri dish was gently rocked to distribute the cells evenly over its surface. The counting, diluting, and plating operations were carried out as rapidly as possible to avoid the formation of cell clumps. The dishes were then exposed to a dose of 2000 rads at a dose-rate of approximately 45 rads per minute. At this dose level, less than one reproductively viable S3 HeLa cell will survive in ten such plates. However, a fraction of the cells will not die immediately but form "giant cells" which have lost their reproductive capacity but retain the ability to metabolize nutrients. These giant cells constitute what has been described by Puck and his co-workers as a "feeder" layer. (22) Presumably,
the function of the feeder layer is to remove small quantities of toxic substances from the medium, thus rendering it more accommodating to cell growth and colony formation; the result is a higher plating efficiency. The plates with their feeder layers were then stored in the incubator overnight and on the following day they were used in the radiation experiment. The reasons for the use of a feeder layer are discussed in Appendix I.

On the following day, a stock bottle was carefully selected, the cells dispersed with trypsin, counted and diluted as already described for the feeder layer. A known number of cells was then inoculated into each dish, such that whatever dose of radiation they were to receive, approximately the same number would survive to form colonies as were expected on the unirradiated control plates which were inoculated with 200 cells. Between 140 and 150 colonies usually formed on the control plates and between 100 and 400 on the irradiated plates. This range allows a meaningful statistical evaluation of the results, and at the same time, avoids excessive overlapping of colonies which would cause difficulty in scoring.

Six replicate dishes were used for each treatment regime.

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1 Plating efficiency is defined as the number of cells that survive to form colonies, expressed as a percentage of the total number of cells plated. Under conditions where the cellular environment is ideal, every cell plated will form a colony and the plating efficiency is 100 per cent.
and at least six were allowed for control. In each experi-
ment, the survival at all three dose-rates was determined for
between one and three total doses. Thus, every experiment
was a self-contained test of a dose-rate effect.

After the cells were plated, they were transferred to
a portable incubator and allowed to attach to the surface of
the petri dish for a period of five to six hours before they
were irradiated. Following irradiation, the cells were
quickly transferred to the "stock" incubator where they
remained for a period of twelve to fourteen days after which
they were removed and the growth medium poured off. The
cells were fixed with a solution of 10 per cent formalin in
normal saline, rinsed in water, and stained with an aqueous
solution of crystal violet. Colonies with more than about
one hundred cells were considered to have arisen from a
single cell that had retained its reproductive integrity and
were therefore counted. To facilitate counting, an image of
the plate, magnified fourteen fold, was projected onto a
screen; this technique made it a simple matter to estimate
colony size. Periodically, plates were counted independently
by two different persons to determine whether any subjective
errors in scoring occurred. Discrepancies were never greater
than about five per cent, and usually less than two per cent.

A dose-rate of 44.9 rads per minute was obtained as
Method of Irradiation

A Picker C10,000 teletherapy unit, containing 6000 Curies of cobalt 60, was used as a source of gamma radiation. Cells were always irradiated inside a portable incubator which could be wheeled into position under the cobalt unit; in this way an ideal environment was maintained for the cells during the exposure. A treatment distance of 120 centimeters was used, the dishes to be irradiated being located in the central portion of the radiation beam within the 98 per cent isodose surface. The plastic top of the petri plus the depth of medium in the dishes was sufficient so that full electron equilibrium was ensured. The geometrical arrangements of irradiation were the same in every experiment. The dose-rates employed in all of the experiments were likewise the same, viz., 44.9, 16.9, and 2.37 rads per minute, and were achieved in two different ways.

1. Filtering the beam

44.9 rads per minute was the dose-rate of the beam filtered only by the top of the incubator. A dose-rate of 16.9 rads per minute was obtained by filtering the beam with an additional one inch of steel, and a dose-rate of 2.37 rads per minute by an additional three inches of steel.

2. Pulsed exposures

A dose-rate of 44.9 rads per minute was obtained as
before, but the 16.9 rads per minute dose-rate was effectively obtained by exposing the cells at 44.9 rads per minute for 22.6 seconds of each minute, and 2.37 rads per minute effectively obtained by an exposure of 31.7 seconds at the beginning of each ten minute period. These exposure times were adjusted slightly to compensate for the "start-stop" mechanical error of the cobalt unit timer.

The doses were measured with a 25r high energy Victoreen thimble chamber whose correction factor was obtained by comparison with an instrument calibrated at the National Bureau of Standards in February of 1963. The exposure dose was converted to absorbed dose by a roentgen-to-rad factor \( f \) of 0.975. An absolute accuracy of \( \pm \) three per cent is claimed for the dosimetry, but the relative accuracy would be expected to be within \( \pm \) one per cent since all three dose-rate measurements were made on the same occasion with the same instrument.

As a check, doses were also measured using the method of ferrous sulfate dosimetry described by Weissbloth, Karzmark, Steele, and Selby (23). The dose in rads is proportional to the change in optical density or absorbance of a solution of ferrous sulfate when its oxidation state is altered upon absorbing ionizing radiation. An 0.8 Normal solution of \( \text{H}_2\text{SO}_4 \), \( 10^{-3} \) Molar in \( \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \), and \( 10^{-3} \) Molar in \( \text{NaCl} \) in a culture dish was placed at the same location as occupied by the cells.
in an actual experiment and exposed to the cobalt 60 gamma source for a known length of time. The change in absorbance (optical density) at a wavelength of 305 milli-microns was measured on a Beckman model DU spectrophotometer by comparing it with a sample of the unirradiated solution. The absorbed dose in rads was then obtained by substituting the appropriate values and solving the formula,

\[ D = \frac{K(\Delta A)}{\sigma l \rho G} \]

where \( D \) represents the absorbed dose in rads; \( K \), a constant equal to \( 3.69 \times 10^{-12} \); \( \Delta A \), the change in absorbance (optical density) at a wavelength of 305 millimicrons; \( \sigma \), the atom-cross-section for absorption at 305 millimicrons; \( l \), the path length of light in centimeters (i.e. the size of cuvette used in measuring absorbance; \( \rho \), the density of the solution; and \( G \), the number of \( \text{Fe}^{++} \) ions converted to \( \text{Fe}^{+++} \) ions per one hundred electron volts of absorbed energy.

The doses measured by Ferrous sulfate dosimetry agreed to within two per cent of the doses measured using the Victoreen high energy thimble chamber.

Method of Evaluating the Results

Survival curves were fitted to the experimental data by a modification of the methods described by Kimball and Gurian. (24,25) A Control Data 160 Digital Computer was programmed by Dr. G. V. Dalrymple, and was used to facilitate the mathematical operations and the associated statistical calculations.
These computer programs are listed, through the courtesy of Dr. Dalrymple, in Appendix II where the application of

Results of the two series of experiments in which the computer in evaluating the results is discussed in
dose-rates were varied by filtering and pulsing the radia-
more detail.

tion are summarized in Tables 1 and 2. From these data, it
is evident that whether a particular dose-rate was obtained
by pulsing or filtering the radiation, there is no signifi-
cant difference in the number of survivors at any given total
dose. Data from both series of experiments were therefore
pooled.

The fraction of colony-forming survivors at each dose
is shown graphically in Figures 1, 2, and 3 where doses were
delivered at rates of 44.9, 16.9, and 2.37 rads per minute
respectively. In each of these three figures, the dose in
rads is plotted on a linear abscissa against the correspond-
ing fraction of cells surviving on a logarithmic ordinate.
The curves drawn in each case are of the kind described by
Gunter and Kohn as "Type C", and have the form

\[ f = 1 - (1 - e^{-D/Do})^n, \]

where \( f \) is the fraction of cells surviving a dose \( D \) rads;
\( n \) is the value on the ordinate obtained when the exponential
region of the curve is extrapolated to zero dose, and \( Do \) is
that increment of dose which reduces the surviving fraction
to \( e^{-1}f \) or approximately 0.37f once the curve has become
EXPERIMENTAL RESULTS

Results of the two series of experiments in which dose-rates were varied by filtering and pulsing the radiation are summarized in Tables 1 and 2. From these data, it is evident that whether a particular dose-rate was obtained by pulsing or filtering the radiation, there is no significant difference in the number of survivors at any given total three dose-rates. The effect is, of course, most striking at the higher dose levels. Figure 4 illustrates three plates B, C, and D which were all inoculated with 20,000 cells and dose. Data from both series of experiments were therefore all received a dose of 1000 rads. The only difference in pooled.

The fraction of colony-forming survivors at each dose treatment between the plates was the rate at which the dose was delivered: 44.9, 16.9, and 2.37 rads per minute respectively. The number of colonies on plates B and D differ by a factor of almost 3/1. respectively. In each of these three figures, the dose in rads is plotted on a linear abscissa against the corresponding fraction of cells surviving on a logarithmic ordinate. The curves drawn in each case are of the kind described by

\[ f = 1 - (1 - e^{-D/Do})^n, \]

where \( f \) is the fraction of cells surviving a dose \( D \) rads; \( n \) is the value on the ordinate obtained when the exponential region of the curve is extrapolated to zero dose, and \( Do \) is that increment of dose which reduces the surviving fraction to \( e^{-1}f \) or approximately 0.37f once the curve has become exponential. (26) The parameters \( n \) and \( Do \) were calculated by a modified least squares technique; the standard error of each value was also calculated (See Appendix II), and are listed in Table 3.
exponential. (26,27) The values of the parameters \( n \) and \( D_o \) were calculated by a modified least squares technique; the standard error of each value was also calculated (See Appendix II), and are listed in Table 3. There is a clear separation between the dose response curves obtained at the three dose-rates. The effect is, of course, most striking at the higher dose levels. Figure 4 illustrates three plates B, C, and D which were all inoculated with 20,000 cells and all received a dose of 1000 rads. The only difference in treatment between the plates was the rate at which the dose was delivered; 44.9, 16.9, and 2.37, rads per minute respectively. The number of colonies on plates B and D differ by a factor of almost 3-1/2.

<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
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<td></td>
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<tr>
<td>0.0410</td>
<td></td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
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<td></td>
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<tr>
<td>Dose (rads)</td>
<td>Fraction surviving at a dose-rate of 2.37 rads per min.</td>
<td>Fraction surviving at a dose-rate of 16.9 rads per min.</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>100</td>
<td>0.698</td>
<td>0.692</td>
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<tr>
<td>200</td>
<td>0.481</td>
<td>0.455</td>
</tr>
<tr>
<td>200</td>
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<td>400</td>
<td>0.233</td>
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<tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>1000</td>
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<td>0.0115</td>
</tr>
</tbody>
</table>
**TABLE 2**

**PULSED RADIATION**

<table>
<thead>
<tr>
<th>Dose (rads)</th>
<th>Fraction surviving at a dose-rate of 2.37 rads per min.</th>
<th>Fraction surviving at a dose-rate of 16.9 rads per min.</th>
<th>Fraction surviving at a dose-rate of 44.9 rads per min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>0.575</td>
<td>0.498</td>
<td>0.510</td>
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<td>400</td>
<td>0.275</td>
<td>0.249</td>
<td>0.214</td>
</tr>
<tr>
<td>600</td>
<td>0.0990</td>
<td>0.0788</td>
<td>0.0596</td>
</tr>
<tr>
<td>800</td>
<td>0.0441</td>
<td>0.0283</td>
<td>0.0215</td>
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<tr>
<td></td>
<td>0.0530</td>
<td>0.0285</td>
<td>0.0208</td>
</tr>
<tr>
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<td>0.0204</td>
<td>0.0125</td>
<td>0.00652</td>
</tr>
<tr>
<td></td>
<td>0.0254</td>
<td>0.00874</td>
<td>0.00558</td>
</tr>
</tbody>
</table>
The fraction of cells surviving various doses of cobalt 60 gamma radiation delivered at 44.9 rads per minute. The crosses (x) represent data from experiments in which the lower dose-rates were varied by pulsing the radiation. The open circles (0) represent data from experiments in which the lower dose-rates were varied by filtering the radiation. The curve is of the kind described as "type c" by Gunter and Kohn, and was fitted to the experimental data by a modified least squares technique (26,28). The value of $D_0$ is 181 rads with a standard error of 16; the extrapolation number, $n$, is 1.60, with a standard error of 0.23.
Fraction surviving vs. dose in rads

- 44.9 RADS/MIN.
- n = 1.6
- $D_0 = 181$ RADS
The fraction of cells surviving various doses of cobalt 60 gamma radiation delivered at 16.9 rads per minute. The crosses (x) represent data from experiments in which the dose-rate was varied by pulsing the radiation; the open circles (O), from experiments in which the dose-rate was varied by filtering the radiation. The curve is of the kind described as "type c" by Gunter and Kohn, and was fitted to the experimental data by a modified least squares technique (26,28). The value of Do is 205 rads with a standard error of 9 rads; the extrapolation number, n, is 1.40 with a standard error of 0.09.
16.9 RADS/MIN.

\( n = 1.4 \)

\( D_0 = 205 \text{ RADS} \)
The fraction of cells surviving various doses of cobalt 60 gamma radiation delivered at 2.37 rads per minute. The crosses (x) represent data from experiments in which the dose-rate was varied by pulsing the radiation; the open circles (O), from experiments in which the dose-rate was varied by filtering the radiation. The curve is of the kind described as "type c" by Gunter and Kohn, and was fitted to the experimental data by a modified least squares technique (26,28). The value of Do is 259 rads with a standard error of 32 rads; the extrapolation number, n, is 1.05 with a standard error of 0.20.
DOSE IN RADS

2.37 RADS/MIN.

n = 1.05
D₀ = 259 RADS
<table>
<thead>
<tr>
<th>Dose-rate (rads per minute)</th>
<th>Do with Standard Error (rads)</th>
<th>n with Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.37</td>
<td>259 ± 32</td>
<td>1.05 ± 0.20</td>
</tr>
<tr>
<td>16.9</td>
<td>205 ± 9</td>
<td>1.40 ± 0.091</td>
</tr>
<tr>
<td>44.9</td>
<td>181 ± 16</td>
<td>1.60 ± 0.23</td>
</tr>
</tbody>
</table>

The pooled results are summarized. A "t" test was applied to the values of Do which indicated a separation at better than the 95 per cent level of confidence between the highest and lowest dose-rates. The Do's at dose-rates of 2.37 and 16.9 rads per minute are separated at the 90 per cent level of confidence; the Do's at dose-rates of 16.9 and 44.9 rads per minute are separated at the 81 per cent level of confidence.
An illustration of the dose-rate effect. Plate A is a control dish into which 200 cells were inoculated; the plating efficiency was 75 per cent. Plates B, C, and D each received 20,000 cells, and were exposed to the same dose, 1000 rads, at dose-rates of 44.9, 16.9, and 2.37 rads per minute respectively.
A B C D
DISCUSSION

181 rads, the value of Do obtained for the survival curve at 44.9 rads per minute, appears high on first impression. Other workers have estimated this parameter to be approximately 140 rads for HeLa cells in culture irradiated with medium voltage x-rays. The present report however, is one of the first in which the parameter has been measured for HeLa cells using cobalt 60 gamma rays, and it has been well established that radiation of this quality is less efficient in damaging biological systems. The Relative Biological Effectiveness (RBE) of cobalt 60 gamma radiation has been determined in a variety of systems and is less effective than 220 kilovolt x-rays by a factor of approximately 0.85. (29) If this factor is applied to the value of Do obtained at 44.9 rads per minute in this investigation, it is seen that a dose of 181 rads of cobalt 60 gamma rays is equivalent in biological effectiveness to about 154 rads of 220 kilovolt x-rays. This is in reasonably close agreement with the value reported by Puck and his co-workers, which is 140 rads when corrected for the fact that the cells were irradiated on glass dishes. (30, 31) Thus, upon further reflection, the value of Do reported here is not only within limits of credibility, but is considered to be an accurate
The ideal experimental method of obtaining a variety of dose-rates would be to alter the distance between the cobalt 60 and the cells being irradiated. The use of this method was frustrated, however, by the fact that the Picker teletherapy unit features a heavy steel counter-weight into which the radiation beam always points. This arrangement, while a good personnel radiation protection measure, tightly restricts the range of dose-rates that can be obtained by altering the subject to source distance. It was decided therefore, with some reluctance, to vary the dose-rate by placing steel filters in the beam. The objection was that excessive attenuation of the primary radiation might lead to the introduction of enough lower energy components by Compton scattering, to alter the Relative Biological Effectiveness of the cobalt 60 gamma photons. Some encouragement toward ruling out an effect of this sort was offered by experiments reported in the literature demonstrating that ten centimeters of water does not measurably affect the RBE of a cobalt 60 beam. (29, 32) The possibility was eliminated by the parallel series of experiments where the dose-rate was varied by pulsing the radiation. Experimental results using this method of obtaining the three dose-rates did not differ significantly from the results obtained by filtering
the beam. In contrast, the difference in values of Do for the survival curves obtained at the three dose-rates is statistically significant, particularly between 2.37 and 44.9 rads per minute where the difference in slope is significant at better than the 95 per cent level of confidence.

It is of theoretical interest to note that as the dose-rate is decreased, either by pulsing or by filtering the radiation, the increase in Do appears to reflect the process of cellular repair described by Elkind and Sutton. They demonstrated that if two or more damaging events must occur within a cell in order to render it incapable of further division, and if cells are given a radiation dose in several separate fractions, sub-lethal damage accumulated during one dose fraction may be partially or totally repaired before a subsequent dose. Consequently, the fractionated doses are less effective in causing reproductive death than when the radiation is delivered in a single exposure. They further anticipate that as the magnitude of each dose fraction is decreased and the period between doses increased, the effectiveness of the total dose will be correspondingly reduced.

In the present investigation when the radiation was pulsed, as well as in the limiting case where the radiation was actually delivered continuously at a lower dose-rate, their prediction was at least qualitatively substantiated. A
quantitative description that enables a prediction of the magnitude of dose-rate effects from a knowledge of the repair process awaits elucidation.

If several assumptions are made, the data obtained in these experiments with single cells in culture may be used to predict the variation in LD 50/30 expected for mice at different dose-rates. These assumptions are as follows:

Firstly, acute radiation death in mammals when the dose is in the range of 400 to 1200 rads is the direct result of a definite fractional depopulation of hemopoietic cells. In other words, whatever the conditions of radiation treatment, the animal will die an acute death if its blood-forming cells are reduced below a definite fraction of the total. Secondly, the response of these cells is essentially the same as HeLa cells grown in vitro. Thirdly, the LD 50/30 for mice exposed to cobalt 60 gamma radiation at a dose-rate of 16.9 rads per minute is approximately 800 rads; this figure is arbitrarily assumed for convenience in calculation. The LD 50/30 is not a constant for mammals and varies widely with species and strain. The absolute value of the LD 50/30 is relatively unimportant in this discussion as long as the figure chosen for calculation is within the wide boundary limits that have been observed for this parameter. What is of interest here is the ratio of the LD 50/30's at various dose-rates. All
three HeLa cell survival curves reported have become simple exponential functions of dose, at doses of approximately 800 rads. It can be seen that in this portion of the curves, the ratio of doses necessary to produce the same fractional depopulation of cells at the three dose-rates is nearly constant and does not vary critically with dose.

From Figure 2 it is seen that a dose of 800 rads, delivered at 16.9 rads per minute, will reduce a population of HeLa cells by a factor of 0.028. In order to obtain an equivalent depopulation when the dose is delivered at 44.9 or 2.37 rads per minute, doses of 730 and 940 rads respectively are required. If our assumptions are correct, these would correspond to the LD 50/30 values at the three dose-rates.

In Figure 5 the predicted LD 50/30's are compared with published data of the variation in LD 50/30 with dose rate. (12 to 19) Most of the sets of data are for mice, one is for rats. The LD 50/30 is plotted on a linear ordinate against the reciprocal of the cube root on the abscissa. This method of plotting the results was suggested by Bateman, Bond and Robertson since it "linearizes" or presents the data in a linear form. (34) No implications concerning this rather unusual plot are made as to a biologically significant mathematical relationship; it is only a method of illustrating a trend in LD 50/30 with varying dose-rates. The heavy line represents
the variation in LD 50/30 predicted from the single cell data. The only conclusion that may be drawn from this Figure is that the predicted variation closely follows the same general trend as whole body irradiated small mammals. The magnitude of variation is sufficient to suggest that the increase in LD 50/30 with decreased dose-rate commonly observed with mice and rats may be fully accounted for at the cellular level.
FIGURE 5

An illustration of the general trend of decreasing LD 50/30 with increasing dose-rate. The heavy line connecting the three circled crosses (⊙) represents the predicted trend in LD 50/30 from single cell data. The other data points represent published data from dose-rate experiments done with mice; one set of points is for rats. The following is a list of symbols used for plotting the data points associated with each investigator(s).

▼ Vogel, et. al. (20)
	× Logie, et. al. (14)
	+ Neal (17)
	□ Daquisto, et al. (15)
	■ Brown, et. al. (16)
	▼ Thompson, et. al. (12)
	• Henshaw, et. al. (10)
	· Kallman, (18)
	○ Kallman, (18)
SUMMARY

S3 HeLa cells were irradiated with cobalt 60 gamma radiation at dose-rates of 44.9, 16.9, and 2.37 rads per minute. Survival curves at each dose-rate were obtained and compared. It was found that the fraction of cells surviving a given total dose increased, with a decrease in the rate at which the dose was delivered. That is to say, the higher the dose-rate the more effective the radiation was in killing the cells.

The data obtained were used to predict the effect of varying dose-rates on the LD 50/30 of whole-body irradiated mammals. The predictions were compared to published data concerning the effect of dose-rate on the LD 50/30 for mice and rats.

REFERENCES


5. The data obtained were used to predict the effect of varying dose-rates on the LD 50/30 of whole-body irradiated mammals. The predictions were compared to published data concerning the effect of dose-rate on the LD 50/30 for mice and rats.


REFERENCES

Where definite page numbers are not given with a specific reference, the message of the text as a whole has been used.


APPENDIX I


To produce a survival curve which quantitatively links the fraction of cells surviving to the radiation dose, it is necessary to employ a range of doses and observe the proportion of cells which survive each given dose.

The proportion surviving varies widely with the dose of radiation given, and consequently many more cells must be inoculated into the dishes exposed to a high dose so that the number of colony-forming survivors will be in the range 100-400 per dish.

This type of experiment is complicated by the fact that even in the absence of radiation, all of the cells plated do not necessarily form healthy colonies. The percentage that do is called the plating efficiency (P.E.). This quantity is calculated from unirradiated control plates into which a known number of cells, say 200, are inoculated. It is then assumed that this "control" plating efficiency will be the same in all the plates of an experiment, regardless of the number of cells inoculated. The fraction of cells surviving a given dose is the ratio of the number of colonies formed to the number of colonies that would have formed had the plate not been irradiated, i.e.,
Fraction of cells surviving = \( \frac{\text{No. of colonies formed}}{\text{No. of cells plated} \times \text{P.E.}} \)

This may not be a valid assumption since the plating efficiency observed for a control dish containing 200 cells may not necessarily be the same for a dish into which, for example, 10,000 cells are inoculated prior to receiving a dose of 800 rads. The irradiated dish contains, in addition to the reproductive survivors, the irreparably injured cells many of which do not metabolically die, but become giant cells. In effect, these sterile cells constitute a "feeder" layer. In an early publication describing the single cell plating techniques, Puck and his co-workers made the interesting observation that low plating efficiencies may be made to approach 100 per cent if a feeder layer was used.\(^{(22)}\)

These feeder layers consisted of about a million cells, irradiated just heavily enough so that the probability of a colony forming survivor was negligible while the yield of giants remained relatively high. From this observation we might expect that the surviving cells in a heavily irradiated dish would be subject to a higher plating efficiency than either the controls or the dishes exposed to relatively low doses which contain a smaller number of cells. For example, let us suppose that the plating efficiency for control dishes was observed to be 50 per cent. In the same experiment the plating efficiency in the dishes exposed to, say,
1000 r may be anywhere from 50 to 100 per cent. The fraction of cells surviving this exposure dose would then be in doubt by a factor of two. The characteristics of the survival curve would likewise be uncertain, and would vary with the control plating efficiency between experiments.

This is exactly what was observed in the early experiments of the series reported in the present investigation prior to adopting the use of feeder layers. Curves A and C of Figure 6 illustrate an example of two successive experiments which exhibited markedly different plating efficiencies; the difference in survival curves is quite apparent. Curve B was obtained from data in later experiments where feeder layers were employed; here the results were much more consistent and repeatable.

A large number of sterile cells are present in all of the plates of an experiment in which feeder layers are used; consequently, the disparity of cellular environment between plates exposed to high and low doses is greatly reduced. With greater confidence, then, one can assume that the plating efficiency is the same within a single radiation experiment.
The effect of a feeder layer on the survival curve. Curves A and C were obtained at the same dose-rate in two successive experiments which exhibited markedly different plating efficiencies; feeder layers were not used in these experiments. Curve B represents the pooled data from several experiments at that dose-rate where feeder layers were employed throughout. The vertical lines through the data in curve B represent the maximum range of values obtained in these experiments.
The data obtained from the experiments consisted of the fraction of cells surviving ($f$) a range of doses ($D$) at each of the three dose-rates tested. (See Experimental Results, Tables 1 and 2).

It was required to fit the data obtained for each dose-rate by a standard multi-hit survival curve of the form

$$f = 1 - \left(1 - e^{-D/Do}\right)^n$$

where $Do$ is that increment of dose which reduces the surviving fraction to $e^{-f}$, or approximately $0.37 f$, once the curve has become exponential, and $n$ is the value on the ordinate obtained when the exponential region of the curve is extrapolated to zero dose. To fit the data to such a curve, estimates of $n$ and $Do$, together with their standard errors, were necessary.

Kimball has described a graphical method for evaluating the constants $k$ and $n$ in the equation

$$S = 1 - (1 - e^{-kx})^n.$$  (24)

Here, $S$ is equivalent to $f$ as we have defined it, $x$ is equivalent to $D$, and $k$ is the reciprocal of $Do$. He defines a variable

$$u_i = \log_e (1 - S_i); \quad i = 1, 2, \ldots, p$$

where $S_i$ is the fraction of cells surviving obtained from...
experimental data, and minimizes \( v \) in the expression

\[
 v = \sum_{i=1}^{p} \left( u_i^n \log_e (1-e^{-kx_i}) \right)^2
\]

with respect to \( k \). Gurian points out that when this method is used, the larger values of \( S_i \) will be weighted too heavily. (25) As a result, the curve will be poorly fitted at lower values of \( S_i \). She suggests a modification of Kimball's method where adjustments are made by introducing a "weighting" variable.

Dalrymple has devised a similar method which was used in this investigation, whereby an "error" function is minimized from a modified least squares approximation of the fitted curves. (28) This method, which involves tedious calculations if done by hand, was carried out with the aid of a Control Data 160 digital computer programmed by Dr. Dalrymple. The program is listed in Chart I. The operations are as follows:

Experimental data is read into the computer in pairs, i.e. \( D_1/f_1, D_2/f_2, D_3/f_3, \ldots, D_{25}/f_{25} \) where \( D \) is the treatment dose and \( f \) is the fraction of cells surviving that dose. The program is written to accept up to 25 pairs of data. Rough estimates of \( n \) and \( D_0 \) are obtained by inspection of the data, and an \( n_{\text{min}} \) (minimum \( n \)), a \( \Delta n \) (arbitrary increment of \( n \)), and an \( n_{\text{max}} \) (maximum \( n \)) are read in together with
a $D_{o_{\text{min}}}$ (minimum Do), a $\Delta D_{o}$ (arbitrary increment of Do), and a $D_{o_{\text{max}}}$ (maximum Do) followed by a small epsilon.

(This epsilon was set at 0.001 for the calculations done in this investigation, and we shall suffice to say that the computer uses the epsilon in comparison logic.) The computer then calculates the sum of the absolute values of the deviations, normalized to percentages, for each datum point from the survival curve determined by $n_{\text{min}}$ and $D_{o_{\text{min}}}$; this sum is termed the "error". Next the error is computed for the survival curve whose parameters are altered to $D_{o_{\text{min}}}$ and $n_{\text{min}} + \Delta n$. Each time an error is calculated, the value of $n$ is increased by $\Delta n$, until $n_{\text{max}}$ is reached whereupon $D_{o_{\text{min}}}$ is advanced to $D_{o_{\text{min}}} + \Delta D_{o}$. Then the error is again computed for values of $n$ from $n_{\text{min}}$ to $n_{\text{max}}$ at intervals of $\Delta n$. The iteration continues until $D_{o_{\text{max}}}$ is reached and errors for all of the combinations of $n$'s and $D_{o}$'s have been calculated. Any desired accuracy may be obtained by choosing sufficiently small values of $\Delta n$ and $\Delta D_{o}$. To avoid wasting time while the computer iterates on $n$'s and $D_{o}$'s yielding high errors, a relatively large $\Delta n$ and $\Delta D_{o}$ may be read into the computer for a preliminary trial to narrow the range of $n$ and $D_{o}$. A second trial with small values of $\Delta n$ and $\Delta D_{o}$ will then give the desired accuracy. A sample of the computer output is listed for illustration in Chart II.
A second program, also written by Dr. Dalrymple, was used in the computer to determine the "Goodness of Fit" by the chi square test, and the standard errors of \( n \) and \( 1/Do \). The standard error of \( Do \) was then calculated from the relationship

\[
\text{Standard error of } Do = \frac{1}{1/Do + \text{Standard error of } 1/Do}
\]

The program is listed in Chart III. (28) Experimental data was read in as before, in pairs, but followed by the previously computed values of \( n \) and \( Do \). The computer output is shown in Charts IV, V, and VI.
CHART I

ROUTINE TO FIT PARAMETERS DO AND N TO \( Y = 1 - (\exp(-X/Do))^n \) BY A MODIFIED LEAST SQUARE TECHNIQUE

\[
\text{FORMAT}(16/(2P20.8))
\]

\[
\text{FORMAT}(10X,5HDO;=;,F16.4,2X,4HN;=;,F16.4////)
\]

\[
\text{FORMAT}(5X,1HY,10X,5HY;HAT,8X,7HY-Y;HAT//)
\]

\[
\text{FORMAT}(3F16.4)
\]

\[
\text{FORMAT}(10X,5HERRO,8X,2HDO,10X,1HN)
\]

\[
\text{DIMENSION DATA(25,2)}
\]

\[
\text{READ} \ I,M,(DATA(I,J),J=1,2),I=1,M
\]

\[
\text{PAUSE 10}
\]

\[
\text{READ} 2,EENMIN,ENSPAC,ENOUT,DSTART,DSPAC,DTERM,EPS
\]

\[
\text{PUNCH 6}
\]

\[
D=DSTART
\]

\[
\text{ER}=0
\]

\[
\text{DO} \ I=1,M
\]

\[
\text{GON}=-\text{DATA}(I,1)/D
\]

\[
Y=1-(1-\exp(GON))**EN
\]

\[
\text{THE DEVIATION IS NORMALIZED TO PERCENTAGES}
\]

\[
\text{GLO}=\text{ABSF}(Y-\text{DATA}(I,2))/\text{DATA}(I,2)
\]

\[
\text{ER}=\text{ER}+GLO
\]

\[
\text{PUNCH} 2,\text{ER},D,\text{EN}
\]

\[
\text{IF(ER-EPS)11,11,12}
\]

\[
\text{PUNCH} 3,D,\text{EN}
\]

\[
\text{PUNCH} 4
\]

\[
\text{DO} \ I=1,M
\]

\[
\text{GON}=-\text{DATA}(I,1)/D
\]

\[
Y=1-(1-\exp(GON))**EN
\]

\[
\text{RO}=\text{ABSF}(Y-\text{DATA}(I,2))
\]

\[
\text{PUNCH} 5,\text{DATA}(I,2),Y,\text{RO}
\]

\[
\text{PAUSE 1004}
\]

\[
\text{GO TO} 100
\]

\[
\text{EN}=\text{EN}+ENSPAC
\]

\[
\text{IF(ENOUT-EN)13,98,98}
\]

\[
D=D+DSPAC
\]

\[
\text{IF(DTERM-D)14,99,99}
\]

\[
\text{PAUSE 1000}
\]

\[
\text{GO TO} 100
\]

\[
\text{END}
\]

\[
\text{END}
\]

TO THE PROGRAMMER:

THE DATA IS READ INTO THE MACHINE AS PAIRS OF DATA

D1./F1.

D2./F2.

ETC.
CHART II

COMPUTER OUTPUT FROM PROGRAM OF CHART I. EXPERIMENTAL DATA OBTAINED AT 44.9 RADS PER MINUTE.

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CHART III

C ROUTINE TO CALCULATE CONFIDENCE INTERVALS FOR TISSUE CULTURE CURVE

1 FORMAT(16/(2F20.8))
2 FORMAT(4F12.5)
3 FORMAT(//'10X,1HN,3X,F12.6,3X,2HD0,3X,F12.6,6H;'/,,17HSTD;ERROR OF;1/DO,3X,F12.6,'/
,3X,F12.6))
4 FORMAT(//'6X,3H;X;;8X,3H;Y;;6X,5HY;HAT,7X,9HY;-,;Y;HAT//)
5 FORMAT(//'10X,10HCHI;SQUARE,5X,F12.6//)

DIMENSION DATA(25,2)

100 READ 1,N,((DATA(I,J),J=1,2),I=1,N)
 PAUSE 10
 READ 2,d,ENN
 SUMUVW=0
 SUMV2=0
 SUMU2=0

DO 10 I=1,N
 U=LOGF(1-DATA(I,2))
 V=LOGF(1-EXPF(-DATA(I,1)/D))
 W=(1-DATA(I,2))*(1-DATA(I,2))
 SUMUVW=SUMUVW+U*V*W
 SUMV2=SUMV2+W*V*V
 SUMU2=SUMU2+W*W

10 SUMUV2=SUMUV2+W*W
 VV=SUMUV2-ENN*SUMUVW
 S2=VV/(N-2)
 PUNCH 4
 A=0
 B=0
 C=0

CHISQ=0

DO 30 I=1,N
 SK=EXPF(-DATA(I,1)/D)
 Y=1-(1-SK)**ENN
 Y1=DATA(I,2)-Y
 Y2=Y1*Y1/Y
 CHISQ=CHISQ+Y2
 PUNCH 2,DATA(I,1),DATA(I,2),Y,Y1
 SKN=DATA(I,1)*SK
 W=(1-DATA(I,2))*(1-DATA(I,2))
 V=LOGF(1-SK)
 GU=Y*SKN
 DR=1-SK
 A=A+W*V*V
 B=B+W*(SKN/DR)*(SKN/DR)
 C=C+W*(GU/DR)

30
CHART III (contd.)

B = E[N]*E[N]*B  
C = E[N]*C  
SK2 = S2*B/(A*B-C*C)  
SN2 = S2*A/(A*B-C*C)  
PUNCH 5, CHISQ  
C = 1/D  
PUNCH 3, E[N], D, C, SN2, SK2  
pause 1004  
GO TO 100  
END  
END  

chi square  0.77223

n  1.050000  do  259.000000  1/do  0.003861

std error of 1/do  0.00036  std error of n  0.137606
CHART IV

COMPUTER OUTPUT FOR DATA OBTAINED AT 2.37 RADS PER MINUTE

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<th>y hat</th>
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chi square | .079223

n | 1.050000 | do | 259.00000 | 1/do | .003861

std error of 1/do | .000543 | std error of n | .197606
<table>
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<th>y - y hat</th>
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chi square  .047859

n  1.400000  do  205.00000  1/do  .004878
std error of 1/do  .000220  std error of n  .090725
### CHART VI

**COMPUTER OUTPUT FOR DATA OBTAINED AT 44.9 RADS PER MINUTE**

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<td>.00558</td>
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<td>-.00079</td>
</tr>
</tbody>
</table>

**chi square** .051983

\[ n = 1.60000 \quad \text{do} = 161.00000 \quad 1/\text{do} = 0.005524 \]

**std error of 1/\text{do}** .000535

**std error of n** .231518