DOCTORAL DISSERTATION SERIES

Publication No.: 21, 357

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University of Michigan

TITLE: THE EFFECTS OF GAMMA RAYS ON STAGES OF ENTAMOEBA HISTOLYTICA

University Microfilms, Ann Arbor, Michigan

THE EFFECTS OF GAMMA RAYS ON STAGES OF ENTAMOEBA HISTOLYTICA

by
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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in the
University of Michigan
1956

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THE EFFECTS OF GAMMA RAYS ON STAGES OF *ENTAMOEBA HISTOLYTICA*

by Curt R. Schneider

The object of these studies was threefold: (1) to measure the effect of gamma rays on the rate of division of *Entamoeba histolytica*, (2) to record evidence of genetic changes attributable to gamma radiation and (3) to compare the lethal dose of radiation for the trophozoite and for the cystic stage. Because of the paucity of standardized methods for experimenting with the entozoic amebae, information about their response to radiation has been lacking. A portion of this study was devoted to developing methods of measuring the physiological response of amebae, based on the requirements for *in vitro* culture of the organisms.

Strains UC and NRS amebae were grown in biphasic egg slant tubes with Stone's Locke overlay. For experiments with trophozoites, pools of amebae were washed and suspensions of known numbers of organisms prepared. Exposures were performed using the large Cobalt-60 source in the Fission Products Laboratory at the University of Michigan. Suspensions were contained in Lusteroid tubes during irradiation. Control suspensions were exposed to the conditions of the radiation cave, but were shielded from the source by a concrete wall. Irradiated suspensions were introduced into egg tubes preconditioned with homologous bacteria. Tubes of UC strain amebae were examined for growth at intervals, until motile trophozoites could be discovered. This was arbitrarily defined as ending the lag phase of growth. Increase in lag phase was compared with the lag phase displayed by controls, and interpreted to reflect death of organisms due to gamma radiation. Following irradiation of NRS strain cysts, serial decimal dilutions of test and control suspensions were made and five tubes inoculated with each suspension. The Most Probable Number calculation was employed (Chang and Baxter, 1955, Am. J. Hyg., 61:121) to ascertain the percentage of control cyst and test cyst survival.

A noticeable increase in lag phase began following exposure to about 100,000 roentgens equivalent physical. A dose of 200,000 rep or more resulted in death as indicated by failure of the cultures to grow. The relation between increasing lag phase and increasing doses of radiation is represented by a logarithmic straight line. Radiation did not cause a change in size of amebae or in their capacity for absorbing

eosin. Recovery of the normal lag phase was shown in the progeny of irradiated trophozoites.

A dose of about 10,000 rep noticeably lowered the number of viable cysts in a suspension. A dose of about 18,000 rep destroyed almost 99% of viable cysts. The relation between increasing cyst mortality and increasing doses of gamma rays is represented by a logarithmic straight line. The relative age of cysts and the relative turbidity of the suspending medium did not influence the rate of cyst inactivation.

It was concluded that (1) measuring the lag phase was a useful but only approximative device for determining the effect of gamma rays on trophozoites, (2) the Most Probable Number calculation was less approximative in determining the effect of gamma rays on cysts, (3) the inactivation of trophozoites and cysts by gamma rays is a reaction of the first order, (4) no evidence of genetic changes in trophozoites was found, and (5) data are presented which suggest that cysts are roughly one-tenth as resistant to gamma rays as trophozoites. The implications of the last conclusion are discussed.

PREFACE

A portion of this study was performed under contract with the Department of the Army.

The sapient advice and encouragement of Professor Richard J. Porter constituted an important stimulus to the prosecution of these studies. His counsel as a scientist was to recognize thought and, as an educator, to value it.

A serious debt is owed Dr. Pearl L. Kendrick for her sympathetic and critical reception of parts of the work in progress. Thanks are also due Associate Professor Fay M. Hemphill for his propaedeutic assistance in matters of statistical treatment.

Finally, acknowledgement is made to John V. Nehemias and Roy Welch of the Fission Products Laboratory for their cheerful and unstinted pampering of a zoologist *ex loco suo*.

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INTRODUCTION

The experiments included in this study were intended to elicit information about the radiosensitivity of the pathogenic amebae of man and monkeys, Entamoeba histolytica Schaudinn (1903).

The decision to employ this animal in radiation studies was not made by considering the facility with which the animal may be handled; observations on E. histolytica grown in vitro do not lend themselves readily to quantitative treatment. It was expected, however, that irradiation of amebae would provide data which would be comprehensible in terms of like data recorded for other protozoa.

Studies in which protozoa have been used as test animals in investigating the actions of electromagnetic radiations have been oriented toward two related fields: (1) quantitative studies to reveal the immediate lethal dose and the delayed lethal dose, using radiations of a given wave length and of varying intensities, and (2) elucidation of the mechanisms by which life processes are disrupted. The literature dealt with here includes work with X-rays of varying wave length and with gamma rays of different origins.

HISTORICAL

I. IRRADIATION OF PROTOZOA

a. Early Work. The first researcher to investigate the action of electromagnetic "waves" on Protozoa was Schaudinn (1899). His report, which has withstood the amplifications of the "modern" literature, was that these organisms as a group "exhibit extraordinary diversity in their behavior toward Roentgen rays." In four species of Sporozoa (Clepsidrina polymorpha, Adelea ovata, Karyolysus lacertarum, and an unspecified coccidian) no effect could be observed. In four species of Mastigophora a definite effect was seen involving cessation of motion after six hours of radiation, and rounding and swelling after ten hours. Death of the flagellates occurred after fourteen hours. In twelve species of Rhizopoda varying effects were noted, ranging from no effect (in certain marine forms) to withdrawal of pseudopodia after two hours of radiation and vacuolization and death with longer exposures. In general, amebae were reported to resist death, the result most commonly seen being a reversible withdrawal of pseudopodia and a rounding up of the cytoplasm. This was particularly noticeable in the Testacea.

It is difficult to estimate the ionization equivalent in modern Roentgen units of the dosages employed by Schaudinn. By interpolation from present data for flagellates and ciliates, it is possible that these were of the order of magnitude of 100,000 r.

Zuelzer (1905) placed a variety of Protozoa in a radia-

tion field produced by radium bromide and watched the organisms' behavior under the microscope. Pelomyxa palustris was seen to round up and cease movement. Other rhizopods (Amoeba limax, Arcella vulgaris, Difflugia pyriformis, and Actinosphaerium eichhorni) performed in more or less similar fashion. But two ciliates (Spirostomum ambiguum and a species of Lionotus) displayed a remarkable resistance. The difference in radiosensitivity between rhizopods and ciliates has since been abundantly substantiated.

Willcock (1904) irradiated a variety of animals grouped as Protista, including several species of Protozoa. Euglena viridis reacted to radium rays by excysting and assuming motility. But a second and smaller strain of the same species displayed no reaction. Radium rays caused Stentor viridis to contract repeatedly but the irritability decreased with repeated exposures. Actinosphaerium died after an hour exposure. But ciliates from frog intestine (Opalina, Nyctotherus and Balantidium) gave no response.

Veneziani (1904) made the curious (and unexplained) report that Opalina ranarum remained "alive" for a longer time when removed from the frog intestine if the organisms had been exposed to radium rays.

b. Irradiation of Mastigophora. Bruynoghe and Dubois (1925) reported that the leptomonad forms of Leishmania tropica retained motility at doses of radium rays which prevented them from infecting mice. These authors found the same phenomenon with Trypanosoma inopinatum from the frog.

This suggests a nuclear focus for sensitivity, although it should be remembered that ability to divide and ability to infect are not necessarily synonymous. Nevertheless, the radiosensitivity of many minute parasites has been reported quantitatively in terms of animal infection. Thus, Halberstaedter (1938) demonstrated that one level of X rays (100,000 r) is needed to provide a direct lethal dose for Trypanosoma gambiense with visible changes in the organisms, while a much lower level (12,000 r) interfered with normal division and infectivity for mice while leaving the flagellates motile. Feldman-Muhsam and Halberstaedter (1946) considered the X ray sensitivity of the flagellated forms and L.D. bodies of Leishmania tropica separately, recording the ability of the organisms to grow in vitro as well as the ability to infect hamsters.

A higher resistance was reported for Trypanosoma cruzi by Emmett (1950). He determined that 51,000 r to 100,000 r produced no visible change and were required to destroy infectivity. However, cultures regained their infectivity for mice upon being allowed to grow in vitro for a month. Thus, excluding the possibility of experimental differences, there may be a species difference in sensitivity between T. gambiense and T. cruzi.

Wave-length may be a factor in producing somewhat disparate results. Thus, Patel (1936) found that exposing Trypanosoma brucei to only 6,000 r of soft X rays resulted in a lowering of infectivity for mice.

Damage to parasitic forms may be determined without infectivity studies when the animals are grown in vitro. Lawrence et al. (1953) found that doses of 32,000 to 40,000 roentgen equivalent physical (rep) of gamma rays resulted in death of Trichomonas foetus while, with doses of 26,000 rep and less, growth occurred. Yet all organisms, regardless of dosage, remained motile following irradiation.

Although Protozoa have been often considered to be the most radioresistant of all microorganisms, the very high doses required to kill them immediately (and upon which the above opinion has been based) are probably also necessary to produce the same result in many cells of higher animals. (Scott, 1937). The median lethal dose was used by Schoenborn (1949) to report the sensitivity of the colorless flagellate Astasia longa; this was between 20,000 and 40,000 r.

Earlier, Holweck and Lacassagne (1931) had attempted to interpret sensitivity in Polytoma uvella (as well as in some yeasts) by identifying organelles within the cell with theoretical "target" sizes which were calculated statistically. This procedure has not received universal acclaim and the "Target Theory" upon which it was based (see Discussion) has been largely superseded by biochemical theories for radiation distress.

Often, the kind of information obtained from radiation experiments has depended upon the growth requirements of organisms and, since these vary, the aspect of results seems to vary. Before adoption of the international Roentgen unit,

equation of the work of different authors was usually precluded. However, the value of qualitative data, if interpreted cautiously, may be permanent. An example is the work of Robertson (1932, 1935a, b). She reported that very weak gamma rays from a radium source had the effect, upon cultures of the flagellate Bodo caudatus, of delaying the entry of the organisms into their "continuous, rapid phase of multiplication," and the irradiated organisms were larger than unirradiated controls after 2½ hours of growth. If cultures were irradiated continuously, there was recovery of multiplicative ability together with retention of the larger cell size. Upon removal of the radium stimulation the rate of division reverted to normal, as did the sizes of individual cells.

c. Irradiation of Ciliata. The relatively larger size of ciliates and the ease with which individuals may be manipulated make them choice organisms for radiosensitivity studies. On the other hand, their resistance may be extreme. Bardeen (1907) was not able to observe any effect of X rays on Paramecium, Urostyla, Oxytricha, Halteria, etc., using the equipment of the day, although Zuelzer (1905) had earlier claimed that Paramecium bursaria was more resistant than P. ambiguum. Hance and Clark (1926) reported that small doses of X rays stimulated Paramecium to a more rapid rate of division for a short time, after which a state of depression was observed. A similar observation was made by E. Schneider (1926) who associated the X rays with an

alkaline pH in the culture medium. Roskin (1929) found that radon exposures induced vacuolization in the cytoplasm of Spirostomum ambiguum.

Other morphological changes in irradiated animals have been seen occasionally but with no degree of regularity. Markovits (1921) used mesothorium irradiation during the division and conjugation of Paramecium to produce what he called "strange, monstrous" forms in which the macronucleus and ultimately the micronucleus seemed to be damaged.

The work of Crowther (1926) is usually considered to have introduced quantitative methods in dealing with the radiosensitivity of Protozoa. Using Colpidium colpoda, he found that a dose of 240,000 r produced a perceptible proportion of deaths in the course of a few hours after exposure. A dose of 360,000 r produced immediate death in a considerable proportion of the population. As the dosage rose between these two values a sigmoid mortality curve was obtained which Crowther interpreted, according to his concept of the "random, discontinuous nature of the rays," as evidence of one-hit inactivation. With doses of less than 120,000 r, no effect was seen.

Under the category of visualization may be mentioned the work of Horning (1937) who irradiated opalinid ciliates from frogs with radium. He recorded a shift in the polarity of the mitochondria which, with time, became dissociated from the vegetative granules against which they usually lie.

The ease with which individual paramecia may be handled

and lines of descent followed have made these animals extremely popular subjects of radiological investigation. However, in light of the fact that the macronuclei of paramecia are highly polyploid (Sonneborn, 1949) and because the nuclear role in determining radiosensitivity is generally assumed, similarities of response between these Protozoa and others should be considered first in terms of analogy rather than homology.

In early work, the absence of a unit of dosage resulted in making accurate comparisons impossible. Baldwin (1920) reported that the presence of vital stains rendered paramecia more radiosensitive than controls. Markovits (1921), Hance and Clark (1926), and E. Schneider (1926) were in agreement that the directly lethal dose for Paramecium was higher than they were able to provide. Dognon and Piffault (1931) were the first to discover the order of magnitude of ionization intensity needed to kill the animals immediately (500,000 r). They reported that a dose of about 250,000 r had no effect. These authors also reported that certain dyes apparently increased radiosensitivity.

Once it was realized that immediate killing required extreme energies, the search for intermediate effects began. Back (1939) ascertained that, when the lethal dose of X rays for P. caudatum (500,000 r) was halved, a lag in rapidity of division was produced, but without visible deleterious effects. This lag lasted through a period (38-48 hours) when four to five divisions could have taken place but did not. However,

given two-thirds to five-sixths of the immediate lethal dose, the animals ceased dividing altogether, and individuals were thus observed which lived for a month or longer without dividing. Size increase or monstrous forms were not seen. Indeed, the organisms began to shrink in size following large fractions of the immediate lethal dose, suggesting a predominance of catabolic processes. Halberstaedter and Back (1943) found that a range of doses could constitute an I.L.D. (200,000 r to 700,000 r). The I.L.D. for individuals only three generations removed from a common parent was always the same, although different for different families. When organisms were treated with colchicine 48 hours prior to irradiation, sensitivity was reduced to about one-half that of controls.

In discussing "artificial" changes in sensitivity, mention should be made of the work of Levin and Piffault (1934a, b, c). They found that P. aurelia became resistant to the immediate killing action of X rays when placed in suspensions of lecithin or cholesterol. Also, Giese and Heath (1948) found that if P. caudatum were sublethally irradiated and then exposed to a sublethal dose of heat they were killed, but that if the same degree of heat preceded the same dose of ionization they were not. They reported that sensitivity to both heat and X rays is much greater in paramecia taken from the logarithmic phase of growth than in those from stationary cultures.

Kimball et al. (1952) reinvestigated the phenomenon of delay of division. They found a retardation following

irradiation, not only in the first mitotic interval but also in later intervals. Recovery of the normal rate of division after six divisions was always observed. They concluded that the delay in the first division corresponded to the inhibition of a stage in mitosis while the later delays were thought to be due to the inhibition of synthesis of some substance necessary for division but originally present in excess. Berner (1942) believed that X irradiation decreased the mineral content of P. caudatum, as shown by ashed preparations, and that recovery from the decrease was just complete at forty-eight hours.

The search for genetic alterations in Protozoa has attracted several workers, although evidence for this class of response has never been as clear-cut as in, say, the yeasts and the bacteria. Kimball (1949a, b) has presented reasons for believing that the reduced "vigor of growth" of post-autogamous P. aurelia, following exposure to phosphorus-32, is evidence of a mutational change in the micronucleus. The loss of "vigor" is thought to be due to the combined effect of mutant genes which produce individual effects too small to be detected. However, the percentage survival and the average number of divisions among surviving paramecia are considered to be measures of non-genetic effects (Kimball and Gaither, 1951).

In support of Kimball's work is the earlier research of Powers and Shefner (1948) who reported that the death rate, following autogamy of irradiated paramecia, increased with increasing doses of radiation. Above 12,000 r the curve

approached 100% death, although complete mortality of ex-autogamous clones was not achieved in their experiments.

Evidence of genetic damage is suggested in the work of Wichterman (1948a). He showed that the mating reaction (clumping of organisms) was both weakened and prolonged by increasing doses of radiation.

The specificity of radiosensitivity in the ciliates has already been pointed out in the work of Halberstaedter and Back (1943), who found that the I.L.D. for clones of paramecia more than three generations removed from a common parent could differ hugely. Wichterman (1948a, b) reported that members of one mating type of P. bursaria were more susceptible to X rays than those of an opposite type. He also found that P. calkinsi demonstrated more radiosensitivity than P. bursaria. It should be remembered that the main difference between these paramecia is their genetic constitution. The claim for genetic effects of radiation in these ciliates may be made, then, with more certainty than for other protozoans.

An interesting sideline of the work of Wichterman (1948a) was the demonstration that irradiation of the "green" Paramecium bursaria with a dose of 300,000 r and above resulted in permanent loss of the zoochlorellae, although some of the paramecia survived up to twice this dosage. In line with this observation is the work of Preer (1948, 1950) who showed that the self-reproducing cytoplasmic particle, kappa, of P. aurelia could be inactivated by X rays.

d. Irradiation of Sporozoa. Until relatively recently the Sporozoa have been ignored as radiological subjects. The coccidians are the most available and the easiest organisms in the class to handle. Albanese and Smetana (1937) irradiated oocysts of Eimeria tenella with X rays and found that 13,500 r of slow radiation rendered organisms ineffective for experimental infection. Doses between 2,250 and 4,500 r seemed to stimulate the activity of the sporozoites, causing an increased production of organisms during the patent period. Waxler (1941) found that irradiated oocysts of E. tenella retained their antigenicity. From 9,000 to 13,500 r prevented the organisms from killing their hosts and also permitted the chickens to survive a subsequent heavy infection of unirradiated organisms. Much lower doses (1,000 to 7,000 r) of X rays were reported by Hauschka (1944) to produce aberrant sporogony in the coccidian Adelina deronis, a parasite of oligochaete worms. However, in this case, the radiation also interferred with host fission processes.

Irradiation of Haemosporidia introduces the problem of methodology. This has often been solved by irradiation the host along with the parasites, with, inevitably, indecisive results. Thus, Zain and Wolf (1943) found that a low dose of X rays (200 r) was not sufficient to inactivate the exoerythrocytic stages of Plasmodium gallinaceum. Rigdon and Rudisell (1945) observed that P. lophurae were injured by X rays in vitro and in vivo, as indicated by the development of subsequent parasitemias in chicks, but that a significant

effect *in vivo* was produced only by a dose very close to the lethal dose for the chick.

More profitably, Bennison and Coatney (1945) succeeded in inactivating trophozoite suspensions of *P. gallinaceum* with 20,000 r but not with 10,000 r. Sporozoite suspensions were inactivated by 8,000 r but not by 6,000 r. Very similar results were obtained by Ceithaml and Evans (1946), whose data indicated that *in vitro* exposure of chicken cells parasitized by *P. gallinaceum* to 30,000 r prevented their increase after injection into normal chickens.

The high resistance of plasmodia to low doses of ionizing radiation was further indicated by the researches of Thompson et al. (1948, 1949) with *P. cathemerium* (in canaries) and *P. lophurae* (in ducklings). Birds with tolerable levels of circulating radioiron offered no evidence of damage to the parasites on the basis of morphology and ability to produce an acute parasitemia upon subinoculation.

e. *Irradiation of Rhizopoda*. Amebae were among the first protozoans to be subjected to the action of ionizing radiations. The early workers (notably Schaudinn and Margaret Zuelzer) have already been mentioned. Free-living amebae in particular present the researcher with an invaluable tool for making visual observations: they stay still, or relatively so. In consequence, evidence of cytoplasmic damage following radiation has been searched for, in these forms, before (and sometimes to the exclusion of) other effects. Thus, Zuelzer and Philipp (1925) produced vacuolization of the

cytoplasm of Amoeba (= Sappinia?) diploidea with radium rays and reported seeing these amebae form syncytial masses together following long exposures. Petchenko (1926) followed the action of radium rays of Vahlkampfia sp. and described two successive effects, (a) a "period of stimulation" in which exaggerated movements prevailed, and (b) a "period of depression" in which movements were slower than normal with the amebae tending to round up. The second stage was followed by a gradual return to normal. The observation was recorded of an irradiated ameba beginning division but unable to complete it, with the divided nucleus recoalescing to form a single cell.

The introduction of the Roentgen unit did much to unify radiological researches of every kind, but comparisons of some of the first instances in which it was employed with similar data of the present day can often cast doubt on the "pioneer" methods of calibration. An instance is the report of Klovekorn and Gaertner (1927) that "fresh-water amebae" did not reveal a distinct inhibition of movement, following X ray exposure, until a total dose of 1,312,000 r had been delivered. After an exposure of 2,190,000 r, some motility was still observed in the animals. These workers did not report following the amebae along to determine whether they were able to divide after such intensities of radiation.

Nasset and Kofoid (1927) incubated a radium needle with a culture of Entamoeba histolytica. The radiation was com-

posed for practical purposes of gamma rays and the authors recorded an apparent stimulation of *in vitro* growth. They did not succeed in sterilizing cultures of amebae with the exposures used but did report finding morphological changes in the nuclei.

Koehring (1940) exposed the giant pond ameba, *Chaos chaos*, to the gamma rays of radon and reported a relatively high resistance which, nevertheless, was less than that of some organisms of other classes (*Blepharisma*, *Paramecium*, small flagellates). The addition of neutral red to the amebae produced an increase in sensitivity.

The extremely provocative work of Schaeffer (1946) must be mentioned here, although its interpretation is problematical. He produced, with X rays, clones of amebae (*Chaos chaos*) which were 60% smaller than normal individuals. In these clones the diminished size was perpetuated for a respectable number of generations (covering four years). Further irradiation of such a "small" clone succeeded in producing, again, organisms which were 60% smaller than the first "mutants." There is probably little question that such a phenomenon represents hereditary alteration. However, *C. chaos* is a highly multinucleate animal, really a syncytium, and it is not impossible that size and number of nuclei are correlated. Random destruction of some of the nuclei, leaving the rest to recover, might account for the observation.

Apart from the early work of Nasset and Kofoid (q.v.) the literature contains but a single reference to experimen-

tation with the radiological response of Entamoeba histolytica. This is the work of Sadun et al. (1950) who stated that 120,000 r failed to completely inactivate amebae growing in Shaffer-Fry and Balamuth media. The normal cycle of population growth was not visibly affected by exposures up to 30,000 r. However, exposures of 60,000 to 120,000 r seemed to inhibit growth. Amebae irradiated with 120,000 r were able to produce as severe intestinal lesions in guinea pigs as were controls.

The object of the present experiments was threefold: (1) to ascertain whether the division rate of E. histolytica were lowered by gamma radiation, (2) to record any evidence of genetic changes which could be attributed to radiation, and (3) to determine the lethal dose of gamma rays for the trophozoite and the cyst stages of the organism.

II. THE PHYSIOLOGY OF E. HISTOLYTICA.

The life cycle of Entamoeba histolytica as followed in vitro has been described by Dobell (1928). The variations performed on the original culture method of Boeck and Drbohlav (1925) have been slight, although other media have been proposed. Dobell and Laidlaw (1926) advocated substitution of the glucose in the medium with rice starch.

Under the conditions established by the biphasic medium (a coagulated egg slant overlaid with Stone's Locke solution) and with 48-hours between transfers, the ameba ordinarily maintains itself in the trophic stage, actively ingesting rice starch particles and growing and multiplying. In some

strains, a certain proportion of cysts is usually present.

The hardiness of the organisms and the extent to which they can withstand manipulation has customarily been undervalued and investigators are always astonished at first at their inability to kill all the trophozoites in a suspension by placing them in a cold room overnight. Nakamura and Anderson (1951) reported that some trophozoites survived refrigeration temperatures for 48 to 96 hours. Indeed, Tsuchiya (1946) stated that trophozoites in fresh stools survived far longer in the refrigerator than at room temperature. Thus, the conditions under which an ameba remains viable and the conditions under which it can grow and multiply should not be confused.

For growth, E. histolytica requires an anaerobic environment (Chang, 1946; Jacobs, 1941). But the organisms may be exposed to aerated fluids without suffering a loss of ability to grow when replaced in a suitable environment. Wykoff (1951) centrifuged ameba cultures for fifteen minutes at speeds ranging from 1,500 R.P.M. to 12,500 R.P.M. without producing significant destruction. Moreover, repeated centrifugings at low speeds had no noticeable effect on trophozoites (Miller and Firlotte, 1948; Nakamura et al., 1953).

The growth progress of amebae which have been placed in culture media at 37°C. has been described by Dobell (1928) and Cleveland and Sanders (1930). Recently, Harinasuta and Harinasuta (1955) have reaffirmed the observation of a lag phase following inoculation of amebae onto fresh slants.

This lag phase is not observed when the tubes have been preconditioned with appropriate bacteria six to twenty-four hours prior to inoculation of amebae, presumably because of the initial presence in such tubes of a suitably negative oxidation-reduction potential as well as the products of bacterial metabolism.

The use of different kinds of medium and different bacterial associates has caused several conflicting reports on the role of pH in the cultivation of amebae. Shaffer (1952) reported that amebae tolerated a range between 6.6 and 7.3, with little or no growth above 7.0. Chang (1948) said that pH 8.0 was a limiting value on the alkaline side, while Blumenthal et al. (1954), utilizing amino acid buffers instead of the usual phosphate buffers, found the best growth occurring at pH 8.5 in monobacterial cultures. Balamuth and Howard (1946) considered that pH did not play an important role in limiting growth in culture. They found an initial drop in pH during the first few hours of growth which was followed by a shift to the alkaline.

Fluctuations in temperature constituted a potential source of variability in experiments. Dobell (1928) considered that lowering the temperature below 37°C. always resulted in abnormalities in the amebae. However, Hopkins and Warner (1946) have reported the results of experiments done at room temperature and at 37°C and have found no abnormalities in the organisms but only a difference in the rates of physiological reactions. Cabrera (1954) succeeded in adapting

strains of E. histolytica to growth at 28°C. He also produced evidence that limited growth could occur as high as 42°C. Tsuchiya (1945) reported survival times of trophozoites in stools ranging from 48 to 96 hours at ice-box temperature.

Cysts have been found to withstand 4 to 5°C. for 40 days (Chang, 1943), with the percentage of mature cysts constituting an important factor in viability.

The information in the paragraphs above suggested that slight variations in pH and temperature, if controlled, might be disregarded in experiments dealing with radiosensitivity.

III. GAMMA RAYS AND X RAYS

Gamma rays and X rays are electromagnetic radiations, emitted in quanta from naturally or artificially excited atoms.

These radiations produce their effect in tissue or in cells by quanta entering the atoms of organic molecules and causing the projection of electrons at high speed. Thus, they are usually called "ionizing" radiations.

The distinction between X rays which are produced in generators and gamma rays which are given off by some radioactive elements has disappeared (Bacq and Alexander, 1955). Gamma rays used to be thought to possess wave lengths which were 10 to 100 times smaller than those of X rays. In recent years, machines have been built which produce extremely hard X rays. Thus the distinction based on wave length disappears.

X rays which can be controlled as to wave length are measured by the roentgen unit. A roentgen is defined as "the quantity of X- or gamma radiation that produces in 1 cc of

air at N.T.P. 1 electrostatic unit of electricity of either charge." (Bacq and Alexander, 1955, p. 15).

Gamma rays originating from radioactive elements or fission products are measured by the roentgen equivalent physical. The rep is defined as "that quantity of radiation which will release in water the same amount of energy as is released by 1 r of X rays." (Bacq and Alexander, 1955).

In the present studies, dosages of radiation are reported in terms of rep. Since the cobalt-60 source does not alter its flux, except by slow decay, the rep doses used here are equivalent to time of exposure as long as the distance from the source is constant.

GENERAL CONSIDERATION OF MATERIALS AND METHODS

I. LUSTEROID TUBES

All exposures of organisms to gamma rays were made in ½ x 3½ inch Lusteroid tubes equipped with metal screw-caps. These were sterilized before use by heating in the oven, at 50-60°C., for a week.

Lusteroid tubes were chosen principally because they offered the smallest tube which could be used and which also had a screw-cap, a requirement made necessary because the source was constantly being used for simultaneous experiments, many involving foods.

It has been determined that, under the influence of gamma rays, Lusteroid provides dosimetry values which are different from those of glass. When ferrous sulfate dosimetry is used, values differ by a factor of 0.55 (Gomberg et al., 1954). This increase in oxidation is thought to be due to oxidizing agents produced on the Lusteroid plastic by the action of the radiation. These additional oxidizing factors do not increase the ionization intensity. Nevertheless, they must be considered as introducing another non-specific killing factor, to be included with general manipulation and temperature alterations.

II. THE CARRIER

Since success in achieving repetitions of experiments depended on insuring that tubes were placed at identical

distances from the outside of the source, and since small differences in distance were supposed to result in large differences in dosage over time, a carrier was designed to hold the tubes. This carrier was employed in all experiments except those in which specimens were placed in the center well of the source.

The carrier consisted of two plates of Masonite, held apart by four 2½ inch wooden dowels. One end of this structure was cut away by a concave arc of 5-inch radius, permitting the rack to be placed evenly against the cylindrical source. One-inch holes were drilled, in nine different arcs and at appropriate distances from the concave edge of the rack, to permit making exposures of known dosage with time. Table 1 presents equations of distance and dosage.

Table 1

Inches from edge	Krep/hour*
1.9	100
2.1	95
2.2	90
2.4	85
2.6	80
2.8	75
3.0	70
3.3	65
3.6	60

*Kiloroentgen equivalent physical

III. THE SOURCE

A source consisting of about three kilocuries of artificially radioactive cobalt-60 provided pure gamma rays for these experiments. A description of this source, in the Fission Products Laboratory of the University of Michigan, and an analysis of its radiation field have been published by Lewis et al. (1954). Briefly, the source consists of 100 ten-inch cobalt-60 rods, encased in aluminum and mounted in a cylindrical holder, three rods thick. The cylinder rides an elevator platform which is lowered, for storage purposes, to the bottom of a 17-foot well of water. For use, the cylinder is raised into the radiation "cave," a room shielded by walls of concrete four feet thick. Specimens may be placed so that they are exposed inside the cylinder or outside.

The masonite carrier was designed to fit against the outside circumference of the cylinder. In a few experiments, exposures were made in the center well of the cylinder.

IV. STRAINS OF AMEBAE EMPLOYED

At the commencement of these studies, one strain (UC) of E. histolytica was employed. This was obtained from Dr. Paul E. Thompson, of Parke Davis Co., Detroit, Michigan, in 1950. It came to him from Dr. William Balamuth, University of Chicago, in 1947. It was isolated by Dr. Enid Rodaniche at the Univer-

sity of Chicago and is of uncertain origin. It has been under continuous cultivation in the present laboratory since 1950. It produces few or no cysts in routine culture but grows extremely well, with the sediment of a single tube producing as many as a million organisms after 48 hours of growth. The strain is agnotoxenic, that is, it includes several bacterial species, at least one of which is unknown (Dougherty, 1953). Proteus vulgaris and several coliform strains are known to be present. These amebae are not infective for guinea pigs, but erythrocytes are ingested in vitro.

The strain of Entamoeba coli employed in some experiments is the SO strain, isolated in 1949 in Evanston, Illinois, and turned over to Dr. Balamuth, who eliminated Blastocystis and some of the original bacterial associates while intentionally adding Aerobacter aerogenes.

For studies involving cysts, the NRS strain (of Dobell) was made available to this laboratory by Dr. S. L. Chang, in May, 1955. This is one of Dobell's "histolytica-like" species, originally obtained from a macaque (Dobell, 1931). It has been characterized by Chang (1946).

V. IRRADIATION PROCEDURE

a. Trophozoites. For each experiment, a pool was prepared from 48-hour cultures, centrifuged at

1000-1500 r.p.m. for two minutes, the sediment restored with sterile Stone's Locke solution at room temperature, the tubes recentrifuged and the supernate again exchanged. The resulting suspension of trophozoites was counted in the hemacytometer and diluted to bring the count to between 20,000 and 30,000 organisms per milliliter.

The quantitated suspension was then distributed in 1.5 ml amounts into the Lusteroid tubes in which exposures were made.

Following irradiation, 1 ml of suspension from each Lusteroid tube was inoculated into a preconditioned egg-slant tube and incubated at 37°C.

b. Cysts. The procedure for irradiating cysts varied only slightly from that employed with trophozoites. Smaller numbers of organisms were used, because less damage due to temperature changes and handling was to be expected. Thus, inocula contained approximately only 5000 cysts per ml.

Since a decimal dilution method for ascertaining radiation effects was used with the cysts, requiring a larger amount of suspension, each Lusteroid tube received 2.0 to 3.0 ml of suspension. Following irradiation, the contents of all tubes which had been exposed at the same distance from the source were pooled for making appropriate dilutions.

c. Controls. A control suspension was prepared

for every experiment. Controls were carried through the same manipulations as test suspensions. During irradiations, all controls were placed in a small open cardboard carton which was deposited on the floor of the corridor of the labyrinthine entrance to the cave. The temperature in this site equaled that of the rest of the cave. The controls were protected from radiation by a four-foot concrete wall which effectively cut down the intensity of gamma rays to a level which mimicked the cosmic ray background and had no biological significance.

VI COUNTING

Counting was done in a Spencer bright-line haemacytometer, according to Paulson's method (1932). The modifications of this method introduced by Griffin and McCarten (1949) were not employed; it was felt that the introduction of complications such as formolizing the specimen (to reduce mucoid clumping of the amebae) would not produce a difference in the counts since strain UC grows without mucoid clumping and it is easy to obtain a reasonably homogeneous suspension of the sediment merely by agitating the tube contents with a plugged 1-ml-pipette with rubber suction bulb attached.

In a few preliminary essays, several counts were made of one tube. This procedure was replaced by making one count per tube per time interval, and

then, later, by making two counts. It was realised that, on the scale employed, slight differences in counts were insignificant. However, the use of two counts instead of one introduced an element of uniformity when the total count was low; at the same time it was feasible from the standpoint of time required for making a count.

However, it is realised that this procedure makes it unwise to try to equate the growth curves of two different tubes; they may resemble each other, but they represent two different populations and cannot be treated statistically as samples from the same population. Balamuth and Wieboldt (1951) have already reported this difficulty in their ameba growth curves.

VII. CULTURE METHODS

a. Media. The routine culture method of Boeck and Drbohlav (1925) has been employed for obtaining all the organisms involved in these studies. Glucose is omitted and a carbon-source provided in the form of rice starch, as suggested by Dobell and Laidlaw (1926). Dutch rice starch was supplied by the Stein-Hall Company of New York and was used throughout the experiments. Normal horse serum was used to enrich the medium for the encysting strain (NR8).

In preparing a Boeck-Drbohlav tube (usually re-

ferred to as a coagulated-egg slant, or simply, an egg tube), eight refrigerated hen's eggs were broken into a Waring blendor. Ringer's solution (12.5 ml per egg) was added. When thoroughly mixed, the material was allowed to settle for ten to fifteen minutes before being dispensed, using a B-D Cornwall automatic pipetting unit, in 2 ml amounts into 6-inch screw-cap glass test tubes. The tubes were slanted without butts and inspissated in the horizontal autoclave, using only jacket pressure (15 lbs.). When the slants had set, 6 ml of Stone's Locke solution (Stone, 1936) were added to each tube with the automatic pipette. All tubes were capped loosely and autoclaved at 120°C. for twenty minutes in bundles packed into glass beakers. Because of insufficient heat distribution through the several Pyrex walls between slant and steam, 100% sterilization is not produced by this process; however, a "hard" coagulation of the egg slant is achieved and a subsequent autoclaving at 120°C. for thirty minutes effectively sterilizes all tubes without producing bubbles and "eruptions" in the smooth surface of the slant. Before the second autoclaving, all caps were screwed down tightly. After the second autoclaving, the overlay takes on a deep straw color due to aqueous infusion of egg from the slant. Such fluid is rich enough to support growth of amebae for a few trans-

fers by itself, but is not dependable for long-range maintenance, due probably to minimum quantities of fat and fat-soluble metabolites.

It has been found that, given good liners in the screw-caps, tubes prepared in the manner described may be stored at room temperature or in the ice-box for two or three weeks without practical loss of fluid volume. Routinely, about 500 tubes were made at a time and utilized within two weeks.

b. pH. The pH of the autoclaved Stone's Locke solution used to wash trophozoites and cysts for experimentation varied between 7.2 and 8.0. Following irradiation, washed suspensions of amebae presented pH values of 7.8 to 8.0, as did controls. The pH of culture tubes before inoculation with bacteria or amebae was 7.4 to 7.6. The washing solution probably introduced a killing factor, since the UC strain seemed to grow best at a pH in the range of that of the culture tubes before inoculation, with a slight rise after 48 hours of growth.

c. Preconditioning. All tubes used in growing irradiated amebae were preconditioned with homologous bacteria. This involved inoculating each egg tube with 0.1 ml of suspension from a 48-hour growth of bacteria alone in egg tubes with rice starch. Occasionally circumstances necessitated using bacteria from a 72-hour culture. Length of time for precon-

ditioning varied within the limits of four to six hours. Tubes preconditioned for only three hours prior to adding amebae gave variable results, and on one occasion unirradiated trophozoites did not grow at all in such a tube.

VIII. TEMPERATURE

Amebae were always handled at room temperature. Irradiations were performed in the "cave" of the Fission Products Laboratory (see above) in which temperature fluctuated from 6 to 15°C. in winter and rose as high as 25°C. in summer. Amebae were exposed in the cave for periods ranging from ten minutes to two hours. In spite of this treatment, control trophozoites displayed a predictable lag phase when subinoculated into preconditioned media. Control cyst suspensions were unaffected by temperature changes for short periods.

EXPERIMENTAL RESULTS

I. IRRADIATION OF TROPHOZOITES OF E. HISTOLYTICA.

a. Introduction. This work was commenced by the writer when he was already familiar with the qualitative aspects of the cultivation and in vitro life cycles of the enteric amebae. However, the early literature is especially unpromising with regard to the successful manipulation of trophozoites outside of culture tubes. There was no ready methodology for pursuing the present kind of study. A "measuring stick" for quantitating results had to be developed.

Methods usually applied to the study of population growth and decline in Protozoa would not apply. Individual paramecia may be followed; Cultures of Tetrahymena, which is a motile organism and fills the tube, may be checked for growth in a spectrophotometer; Free-living amebae may be grown on an agar surface in a moist chamber and "colonies" may be watched; but none of these easy methods can be applied with the enteric amebae which grow in limited numbers, under strictly anaerobic conditions, in the sediment at the bottom of an egg-slant tube.

A solution to the problem is presented in the third section of this chapter. It is held, by the writer, to be an inferior solution. However, it permitted extraction of some data from the subsequent

experiments.

b. Normal Curves. To obtain uniform suspensions of organisms, several 48-hour cultures of UC amebae were pooled, twice centrifuged at 1000-1500 r.p.m. for two minutes, and thrice suspended in sterile Stone's Locke solution at pH 7.6 - 7.7. Counting was done in the hemacytometer and further dilutions were made so that 1 ml of suspension contained 10,000, 100,000 or 1,000,000 amebae per milliliter. Each growth tube received 1 ml of suspension. All tubes were preconditioned by adding 0.1 ml of a 48-hour growth of homologous bacteria two or three hours before introducing amebae. Growth was followed at eight or twelve hour intervals thereafter by resuspending the entire sediment of a tube and counting a sample in the hemacytometer. Results were plotted on logarithmic paper as a function of numbers of amebae in thousands per milliliter and time in hours after inoculation.

It had been predicted that a ten-fold difference in the size of the inoculum would be reflected in the subsequent growth curve as a difference in time of lag phase or as a difference in the shape of the curve. Figure 1 presents three growth curves selected from among the tubes receiving 10,000 amebae. The period of logarithmic rise was apparently less subject to variation, given a uniform inoculum, than

the peak and decline of the population.

Figure 2 compares the growth curves of tubes which had received inocula of 10,000, 100,000, and 1,000,000 amebae. These curves represent single tubes and were selected from among many as typical. In general, the impression was gained that the peak of growth is reached earlier with larger inocula but that the absolute height of the peak is variable and is greater when the amount of rice starch is greater. The slope of the logarithmic rise seemed somewhat more depressed as the inoculum was diminished, but this difference was so slight and so variable that it was considered to be without value for suggesting the size of the original inoculum.

The length of the lag phase, i.e., the time elapsing before one ameba could be counted in the suspended sediment, was felt to represent the surest criterion for estimating the proportion of viable amebae in the original inoculum. On the basis of the above observations, it was decided to perform irradiation experiments using suspensions of 20,000-30,000 amebae per milliliter as inocula.

c. Effect of Rice Starch. That an excess of rice starch can have the effect of prolonging the amebic lag phase was recognized by Spingarn and Edelman (1950). This factor was recognized as a variable of potential significance in the present

FIGURE 1
NORMAL GROWTH OF STRAIN UC

(INOCULUM = 10,000 AMEBAE / TUBE)

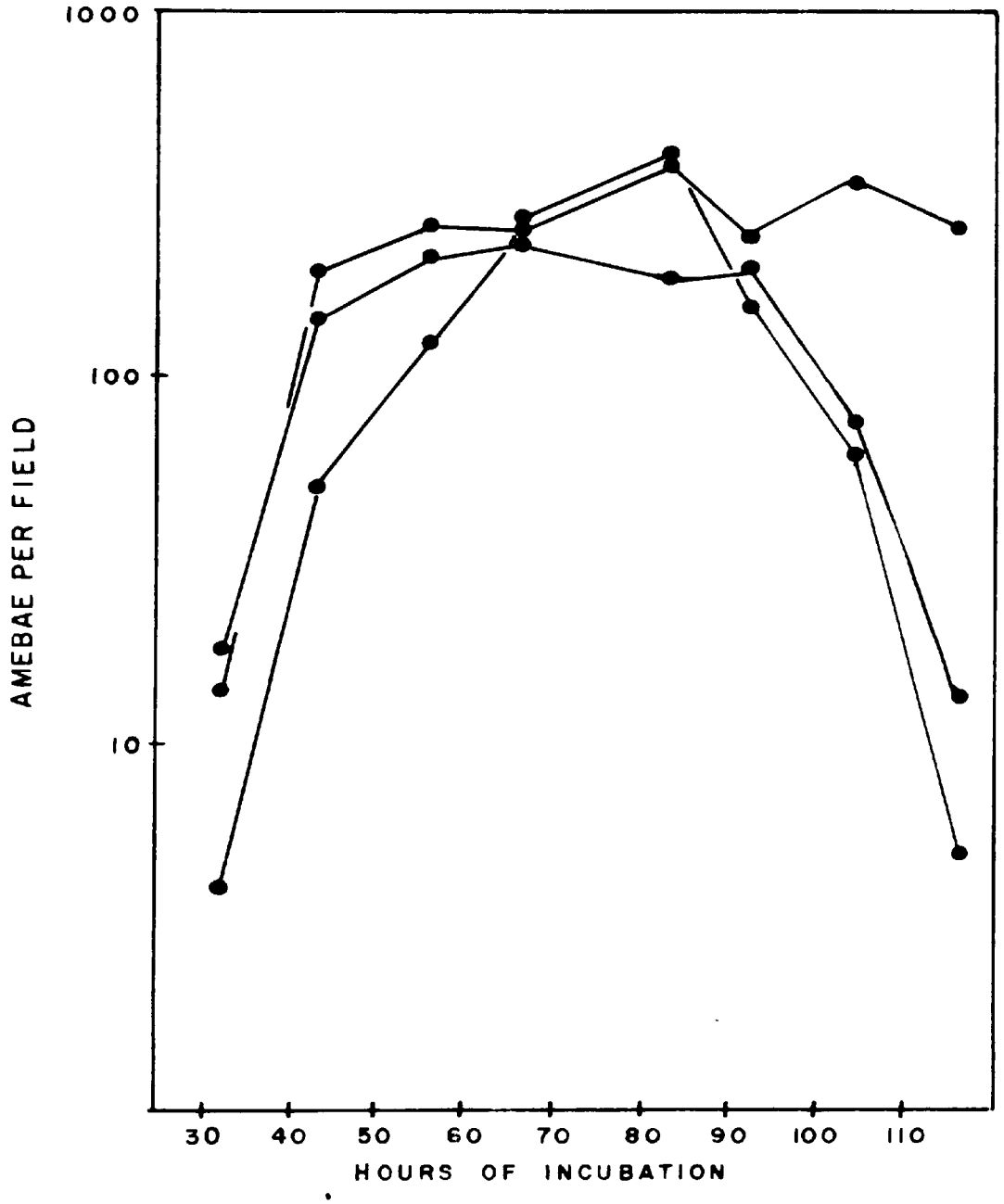
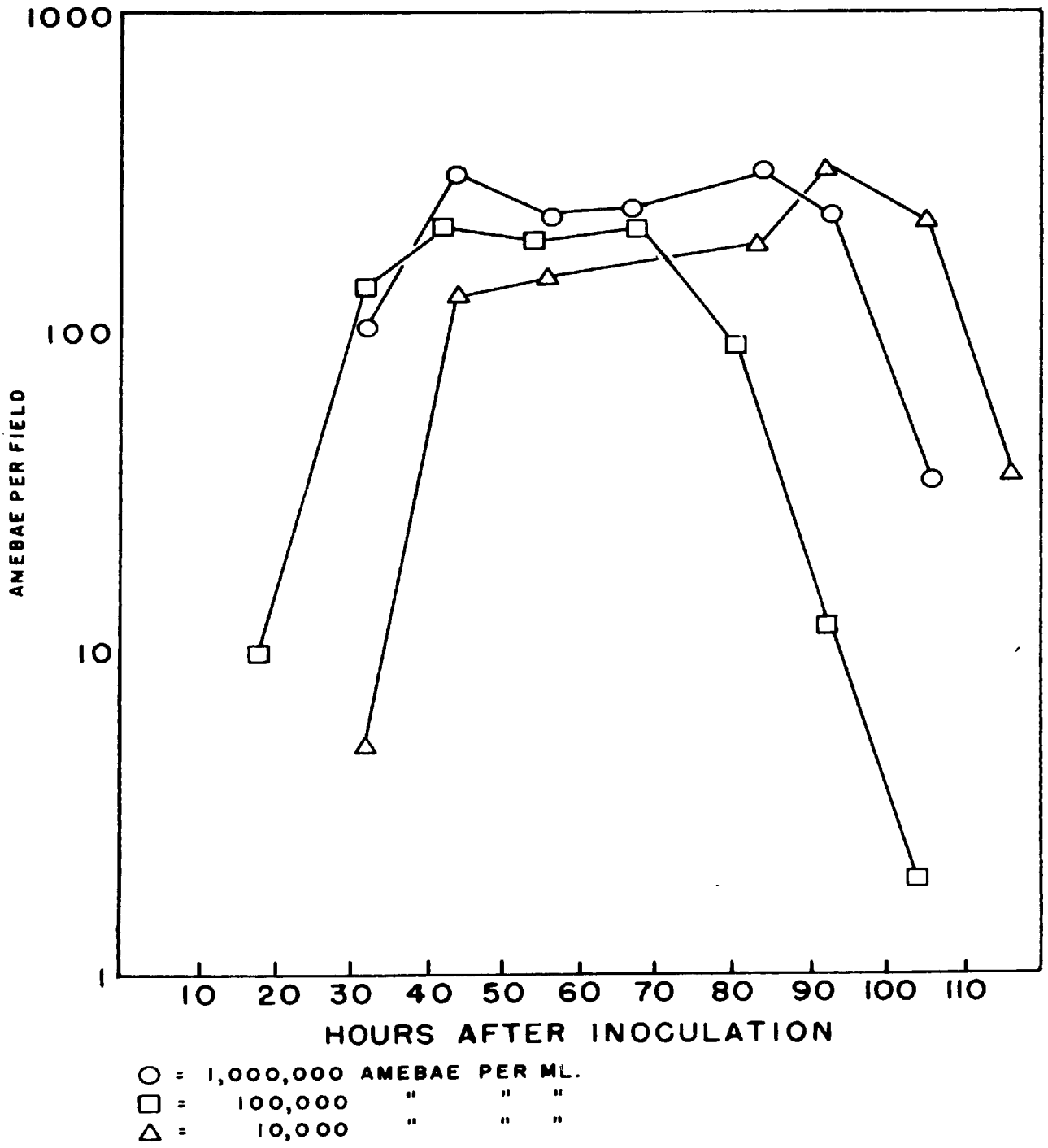


FIGURE 2

NORMAL GROWTH OF AMEBAE FROM INOCULA OF DIFFERENT SIZES



experiments. Accordingly, tests were performed to ascertain the increase in lag phase that would be produced by adding the largest amount of rice starch practicable under the conditions of the experiments. This amount was about 35-50 mgm. Controls received the usual amount of starch used routinely, or about 10-20 mgm.

UC amebae were pooled after 48-hour growth and twice washed in sterile Stone's Locke fluid. The suspensions were counted and the sediment diluted to bring the count to about 10,000 amebae per ml. One ml was introduced into each of eight preconditioned egg tubes. To four of the tubes, 35-50 mgm of rice starch was added, while to each of the four others 10-20 mgm were added. Counts were made at about twelve hour intervals.

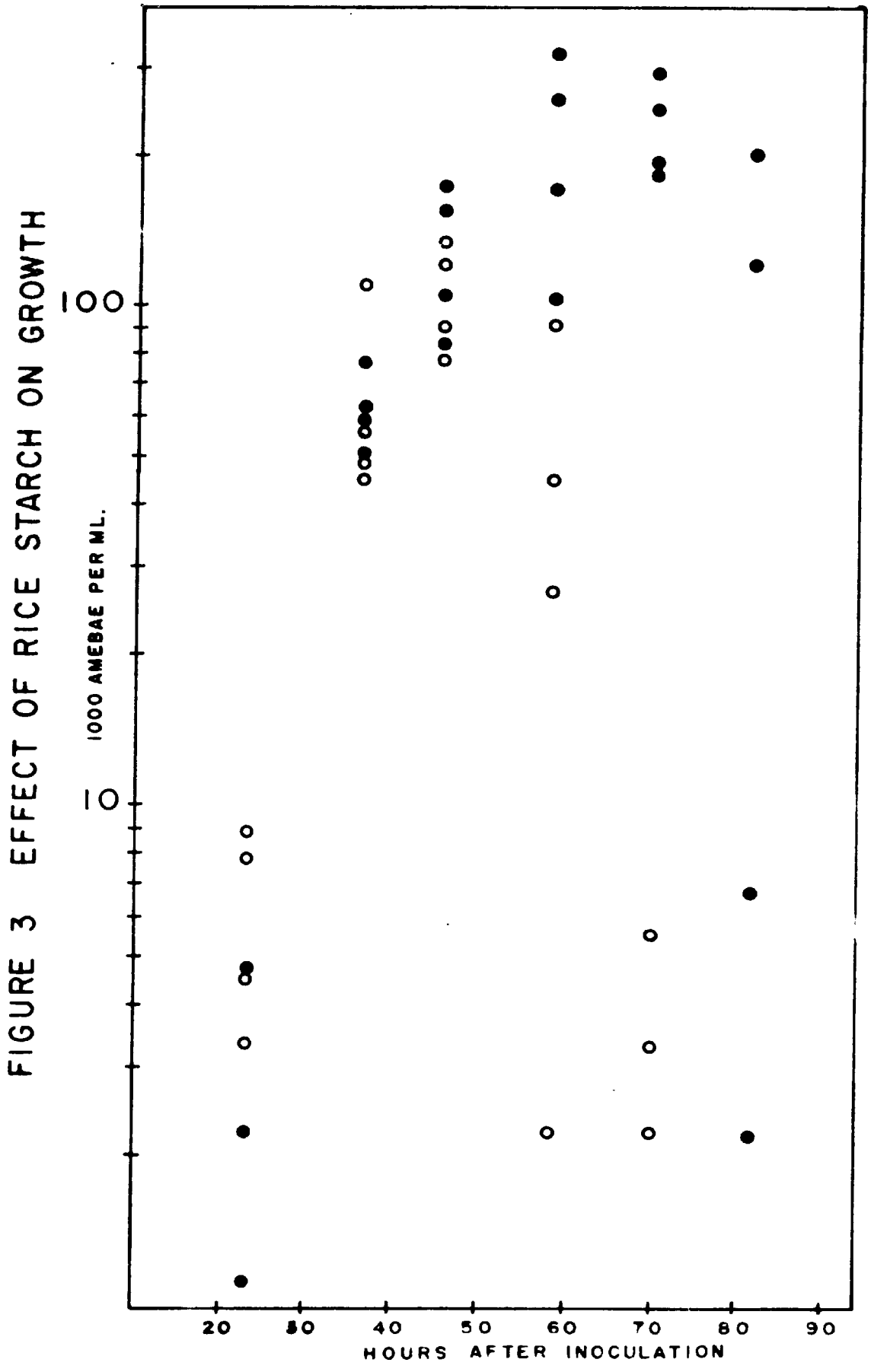
Figure 3 indicates that the populations in tubes with more and with less rice starch group themselves together during the period of logarithmic increase. Thereafter, those tubes receiving more starch maintain their peaks for about ten hours longer than tubes receiving less starch. Spingarn and Edelman (1950) reported different absolute values for the same kind of phenomenon.

On the basis of the uniformity of rise of the populations regardless of the amount of rice starch it was decided that variation in the amount of

starch used routinely would not seriously affect the length of the lag phase in such a way as to conceal an increase in lag phase due to some other cause. The amounts of rice starch used by Spingarn and Edelman (1950) to produce a marked lengthening of the lag phase were 200 to 400 mgm, notably in excess of the amounts used here.

d. Radiosensitivity of Trophozoites. Amebae were exposed to a source consisting of about three kilocuries of cobalt-60, receiving dosages ranging from 135 to 210 kilorep. Exposures were made over a six-month period. Multiple tubes were exposed at the same dosage in each experiment. Controls were placed in the labyrinthine entrance to the radiation cave and were subjected to the same temperature as the test amebae but were separated from them by a four-foot poured concrete wall. The temperature in the cave ranged from 6 to 10°C.

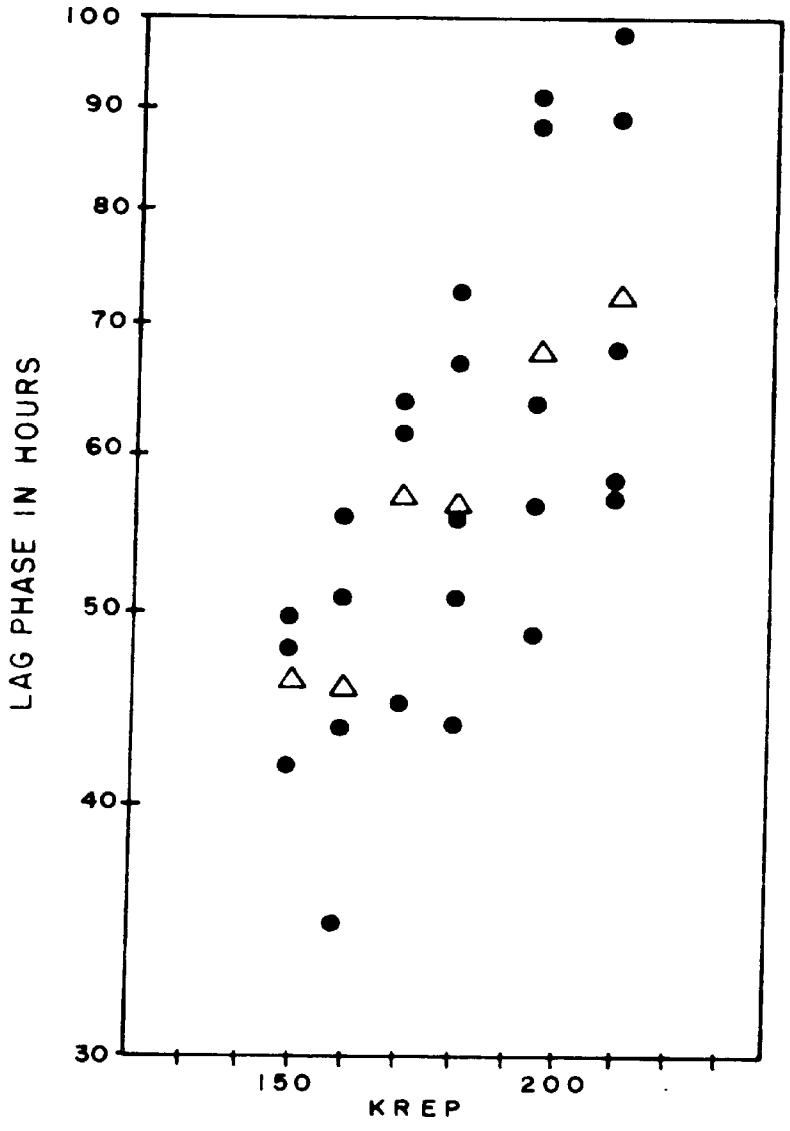
Following exposure, one milliliter of suspension from each irradiated tube was transferred to a preconditioned egg tube. The growth curves were found by counting at eight to twelve hour intervals. Length of lag phase was arbitrarily taken to extend to that time (in hours after inoculation) when the population reached a density of 1000 amebae per ml. End of the lag phase was plotted as ordinates, using radiation dosages in kilorep as abscissal values.


FIGURE 3 EFFECT OF RICE STARCH ON GROWTH
100
10
1000 AMEBAE PER ML.
20
30
40
50
60
70
80
90
HOURS AFTER INOCULATION
O = 10-20 MGM STARCH
● = 35-50 MGM STARCH

In figures 4 and 5, each point represents a single tube. Plotting the points on semi-logarithmic paper produced a scatter graph through which a straight line may be fitted by eye. Under the conditions of the experiments it is believed that this line indicates a true relationship between gamma ray dosage and lag phase. Thus, by extrapolation, it may be interpreted to represent the relation between the dosage and the survival of amebae. The values on the graphs probably have little absolute value. However, the slope of the rise seems to be stable. It will be seen from figure 4 that no values for lag phase have been included after 210 Krep. Beyond this dosage amebae were never found to be able to establish a population. It was also found that the lag phases of amebae receiving 100,000 rep were largely indistinguishable from those of controls. Thus there is a relatively small range between the dose at which no effect was observed and that at which no amebae survived. Amebae are apparently able to resist radiation insults up to doses of 100 Krep. Beyond this level the proportion of amebae unable to recover increases logarithmically. The "extinction point" (at a level of about 210 Krep) may not necessarily represent the dose at which all the amebae are irreversibly damaged but it does represent a point at which the survivors, if any, are few enough

FIGURE 4

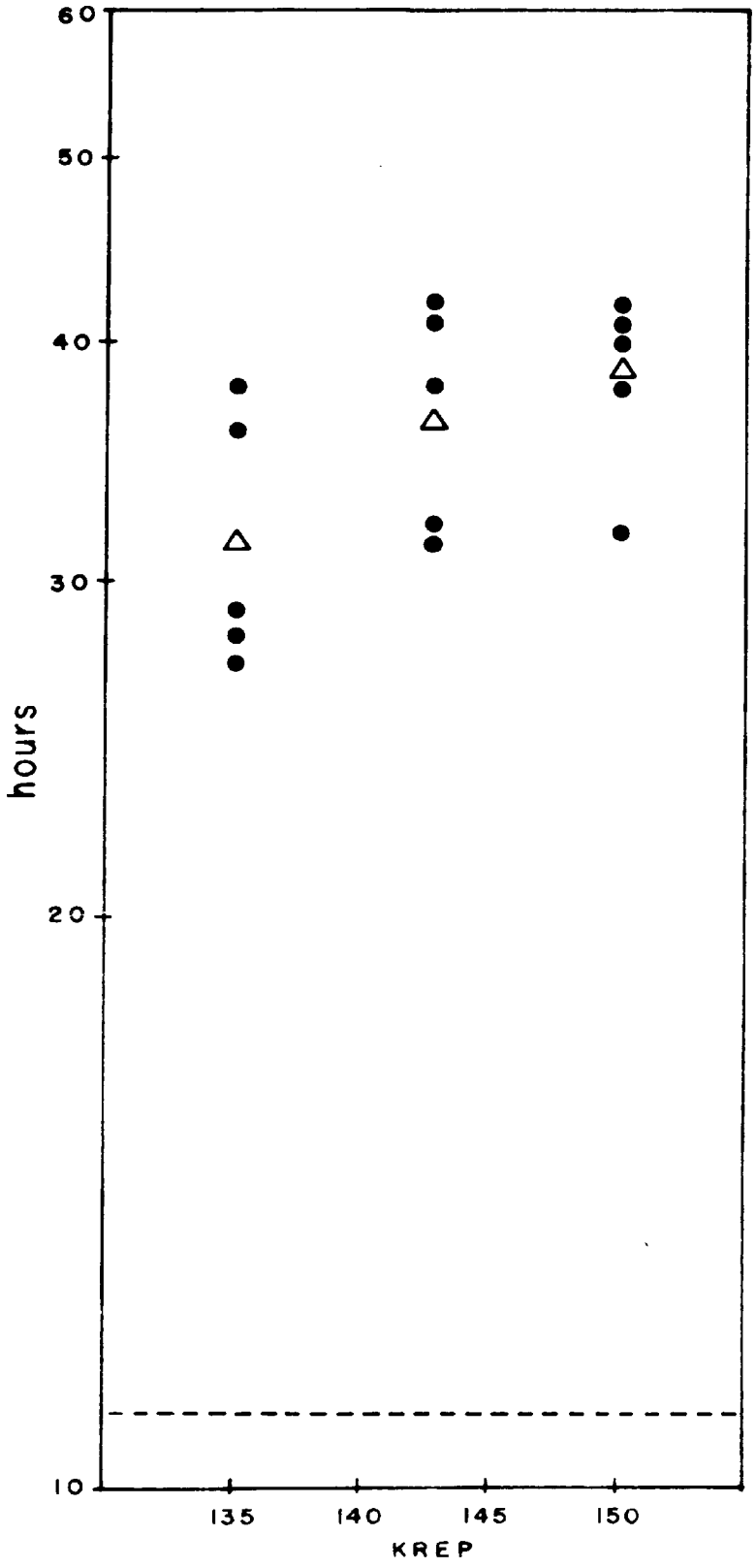
INCREASE IN LAG PHASE WITH GAMMA RADIATION



△ = Antilog Mean

FIGURE 5 INCREASE IN LAG PHASE WITH GAMMA RAYS

(DATA FROM SIMULTANEOUS EXPOSURES)



Δ = Antilog Mean

Dotted line = Control Mean Lag

to be unable to reestablish the population. Brackett and Bliznick (1947) found that as few as 500 amebae in association with organism t produced positive cultures, and occasionally as few as fifteen amebae were sufficient.

e. Effect of radiation upon the size of amebae.

A suspension of UC trophozoites was exposed to a lethal dose of gamma rays (210 Krep). A control suspension was placed at the same temperature as that of the irradiated amebae but was shielded from the rays. Following exposure, samples of the irradiated and control amebae were examined with a calibrated microscope, on a warm stage maintained at 37°C. Ten amebae from each suspension were chosen at random and measured with the ocular micrometer.

The mean diameter of irradiated amebae was 17.66 μ and that of the control amebae was 19.26 μ. The standard errors of these means were respectively 1.11 and 0.83. The means overlap at the lowest level of confidence. The selection of specimens for counting was made at random and it was evident that there was nothing about the amebae in either group which made them easier to see or measure than those of the other group. Table 2 presents these values.

It was concluded that the difference in the means of the diameters of irradiated and control amebae was not statistically significant and that radiation

Table 2

Sizes of irradiated and control amebae. (in micra)

Control	Irradiated
14.4	10.8
16.2	14.4
18.0	16.2
19.8	16.2
19.8	16.2
19.8	18.0
19.8	18.0
19.8	19.8
21.6	21.6
23.4	25.2
$\bar{X}$ = 19.26	$\bar{X}$ = 17.66
SE = ±0.87	SE = ±1.11

had not influenced the size of the organisms.

f. Eosin test. Eosin is a supra-vital stain which has been reported to color only dead or moribund amebae. Living trophozoites are revealed as "white" bodies against a pink background while dead trophozoites show a darker pink or red against the lighter background (Beemer, 1947).

Amebae which had been exposed to 60,000, 90,000, 100,000 and 150,000 rep were stained. Controls had been placed in the cave corridor in which an ionization counter measured activity of about 0.15 rep. This dosage is practically indistinguishable from the background and was thought to be biologically insignificant. The temperature at the time of irradiation was 6°C.

According to the eosin test, amebae were not killed immediately by radiation. Both controls and irradiated amebae revealed about 50% unstained trophozoites out of one hundred counted at random. Dead amebae in the control suspensions were presumed to have suffered from manipulation and cold. It was concluded that irradiation alone did not produce changes detectable by the eosin test.

Yorke and Adams (1926b), using the eosin test with cysts, made an observation which can also be applied to trophozoites; they stated that, while those cysts which stained with eosin were certainly

dead, it was more doubtful whether all those which did not stain were alive. The method also includes the difficulty that a kind of "selection" operates, whereby the stained amebae are easier to see and, therefore, more likely to be counted than unstained amebae. However, since the test was inconclusive, and is considered of doubtful significance by many workers, it was not pursued.

g. Comparison of the growth of progeny of irradiated and unirradiated amebae. Suspensions of strain UC (E. histolytica) and SO (E. coli) which had received doses of approximately 140,000 rep were subcultured for about four weeks. Culture medium and transfer procedure were similar for all strains. The preparations for the experiments were identical with those described for other irradiation runs; also, in preparing suspensions for irradiation, care was taken to bring the counts of the normal (control) amebae into line with those of the suspensions of the progeny of irradiated amebae (designated UC-R and SO-R).

No significant difference was observed between the shapes of the growth curves of UC-R and UC strains (Figure 6) or of SO-R and SO strains (Figure 7). The close resemblance between the curves for UC-R and UC requires little comment. Each line represents the logarithmic mean of five tubes which

were grown simultaneously. The SO-R strain seems at first glance to achieve larger populations, on the average, than the normal SO strain. However, the population counts were very low (compared with the UC-R and UC strains) and were felt to be untrustworthy. The standard errors of the means for each point (representing the mean population from five tubes) overlap each other at a 68% level of confidence (1 $SE_{\bar{x}}$), except for the counts taken at 36 and 46 hours. Thus, statistically there is no indication that the differences observed are not due to chance.

These data suggest that recovery of the normal growth curve had occurred after four weeks of subcultivation of irradiated amebae. The fact that, in the logarithmic phase of growth, progeny of irradiated amebae seemed to achieve higher numbers of organisms may or may not be significant and requires further study.

It should be added that visual inspections of the cultures, necessitated at regular intervals of the experimental growth phase, revealed no notable differences in the morphology of the irradiation progeny and the control amebae.

II. IRRADIATION OF CYSTS OF E. HISTOLYTICA

a. Introduction. As indicated in the preceding pages, the conviction has been acquired that the

FIGURE 6

GROWTH OF IRRADIATED AND UNIRRADIATED AMEBAE (PROGENY)

UC STRAIN

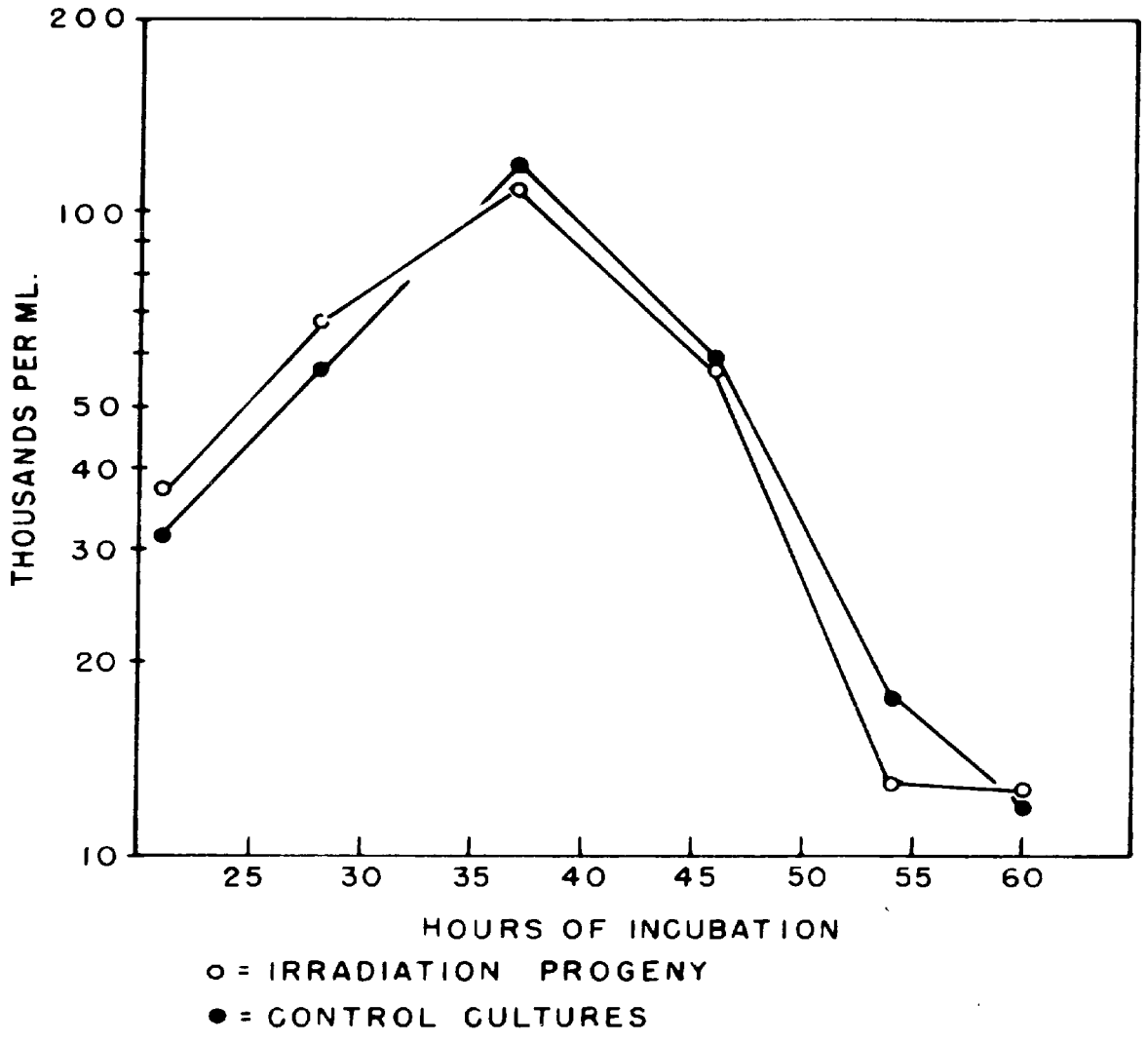
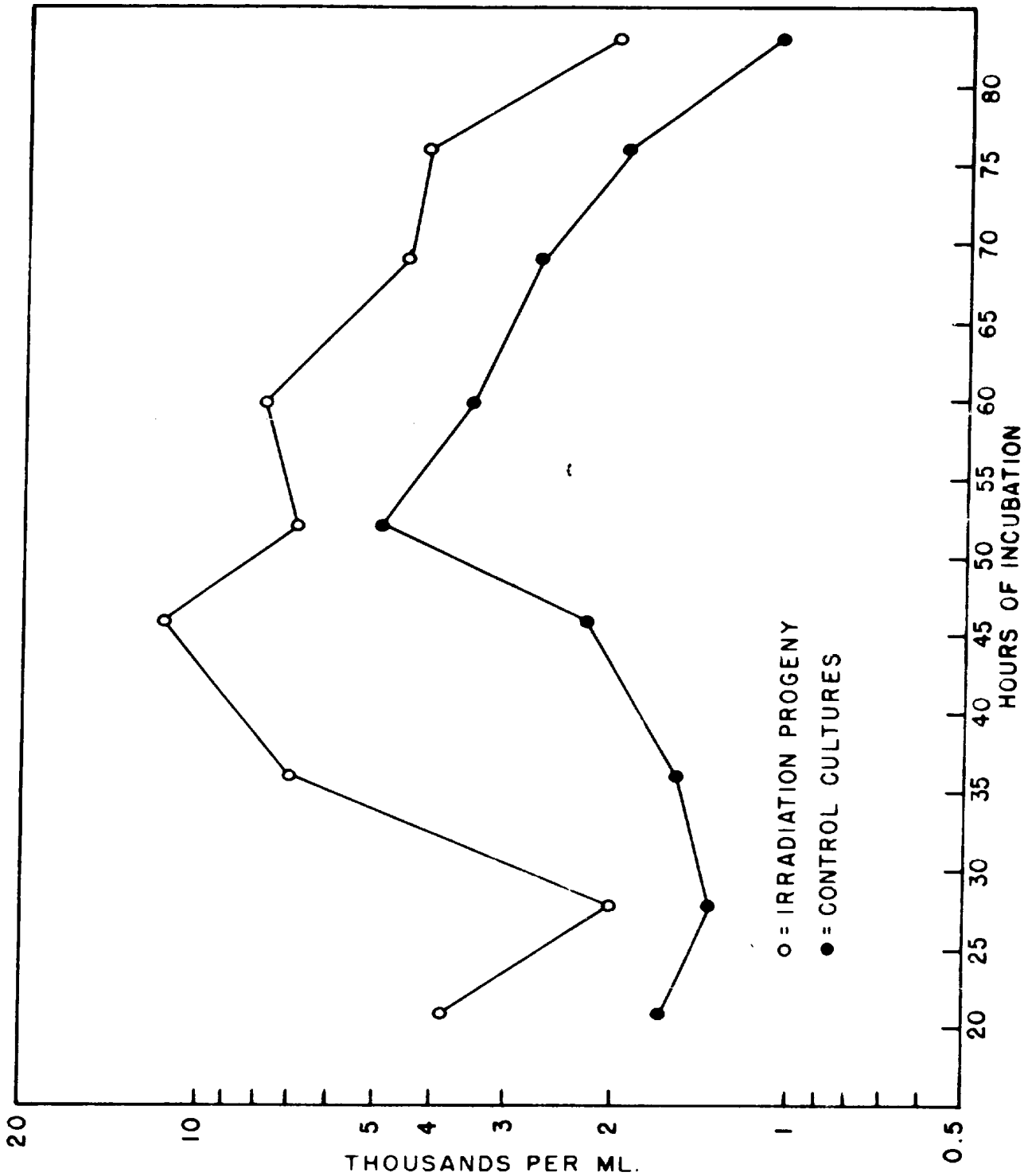


FIGURE 7
GROWTH OF IRRADIATED
AND UNIRRADIATED AMEBAE
(PROGENY)
SO STRAIN



inactivation of trophozoites by gamma rays may be described as accellerating exponentially with time in a constant radiation field.

The egg tubes from which test organisms were harvested represent a highly artificial environment when compared with the gut lumen and tissue spaces of a host. The cyst stage of E. histolytica, on the other hand, offers several experimental advantages over trophozoites. Cysts are fitted to survive the comparative rigor of the external environment and may therefore be investigated under conditions less inimical for the organism. Also, since the metabolic condition of the cyst is putatively at variance with that of the trophozoite, a difference in radiosensitivity might be expected.

From the practical standpoint, a knowledge of the radiation response of amebic cysts will find an application in relation to the sterilization of food with gamma rays (Lawrence et al., 1953; Brownell et al., 1953).

b. Special materials and methods. Diverse methods and media have been reported as successful in promoting in vitro encystation (Dobell, 1926, 1928; Cleveland and Sanders, 1930; Stone, 1935; Chang, 1942; 1945; Kessel et al., 1944; Balamuth, 1951). None of these procedures succeeded with the UC strain. Accordingly, a strain of E. histolytica

(NRS) was obtained which produces cysts in relative abundance during the course of growth in routine egg tubes. The NRS strain was employed in all subsequent experiments.

Eight tubes of NRS amebae were maintained routinely with 48-hour transfers. Rice starch and horse serum (0.5 ml) were added to each new tube at the time of transfer. Transfers were made by agitating and suspending the rather mucoid sediment, using a sterile pipette, and withdrawing 0.5 ml of the culture fluid. This was introduced into a new tube. Tubes from which transfers had been made were replaced in the incubator and left for another 24-hour period. Cysts were harvested from the resulting 72-hour cultures by pooling the contents of the eight tubes into two 30-ml centrifuge tubes, centrifuging at 1000-1500 r.p.m. for 4-5 minutes, discarding the supernates, combining the sediments, washing in 10-15 ml sterile distilled water, recentrifuging for the same time, again discarding the supernate and restoring the volume to 5 ml with sterile distilled water. Examination of the suspension after this treatment showed many trophozoites in the process of lysis and many cysts which seemed undamaged. Iodine staining revealed a good proportion of quadrinucleate cysts, a few binucleates and a large number of uninucleates. The suspension was placed in the cold

at 4-6°C. overnight and always used the next day.

Dobell (1928) has stated that only mature (i.e., quadrinucleate) cysts will hatch. Since cyst suspensions were precious and generally contained barely enough cysts with which to work, the practice of Rees et al. (1950) of discarding harvests showing less than 50% quadrinucleated cysts was not followed. Rather, a control suspension was run with each experiment to determine the "normal viability" of the individual harvest. It was felt that this number really represented a measurement of the proportion of quadrinucleated cysts in the suspension.

Prior to actual irradiation the cysts were centrifuged to remove the distilled water supernate and replace it with sterile Stone's Locke solution. Counting of the suspension before testing was done in the hemacytometer. Sterile Stone's Locke solution was used to dilute the suspension until six different counts gave an average of approximately 5,000 cysts per milliliter of fluid.

Following irradiation, cysts were introduced into egg tubes which had been preconditioned by adding 0.1 ml of a 48-hour growth of homologous bacteria to each tube. Rice starch (10-15 mgm) and horse serum (0.5 ml) were added. The bacteria were allowed to grow alone in the tubes for four to six hours before introducing the cysts, in order to

establish a low oxidation-reduction potential conducive to excystation.

c. Quantitative determination of the survival of irradiated cysts. The suspension being irradiated contained a known number of cysts as ascertained by the hemacytometer. In practice, only about 10% of the healthy-looking cysts were really viable, i.e., capable of excystation under the conditions of the present experiments and establishing a colony. This percentage of viability fluctuated (see below) but of 28 experiments with controls, the controls presented a mean viability of 9.1% as calculated by the Most Probable Number method, and a standard error of the mean of 0.74%.

Since determination of the survival of individual irradiated cysts was impractical, Chang's method of calculating the Most Probable Number of survivors, was employed. (Chang, and Baxter, 1955). This equation is a modification of the approximation equation employed in water analysis for computing the MPN of coliform organisms. The equation is as follows:

$$\text{MPN of surviving cysts/ml of test fluid} = \frac{(\text{total \# pos. tubes}) \times (\text{\# cysts per ml test fluid})}{2\sqrt{(\text{Total \# cysts in neg. tubes}) \times (\text{total \# cysts in all tubes in the series})}}$$

A sample set of data with their MPN calculation would be worked out as follows:

Three sets of five tubes each are employed. The suspension being tested has been adjusted, following irradiation, to a density of 50 cysts per ml. Serial decimal dilutions of the suspension are prepared to give suspensions of 5 cysts/ml and 0.5 cysts/ml. Obviously, in the latter case, the number 0.5 indicates an even chance of withdrawing or not withdrawing a single cyst with a milliliter of fluid.

One milliliter of each dilution is inoculated into each of the five tubes in a set. After 48 hours of incubation the sediment of each tube is inspected for motile trophozoites. All tubes are followed for at least six days before discarding them as negative. Tubes are discarded immediately, however, if they are positive.

The final results of one experiment, representing the controls from a radiation experiment were as follows:

Tubes receiving 50 cysts/ml-5 out of 5 positive.
Tubes receiving 5 cysts/ml-2 out of 5 positive.
Tubes receiving 0.5 cysts/ml-0 out of 5 positive.

Omitting from the calculation the tubes with the largest inoculum which were all positive, the total number of positive tubes was two. The suspension, which served as a control in the irradiation experiment, contained 5,000 cysts per milliliter. There were eight negative tubes, three of which had received 5 cysts each and five of which had received

0.5 cyst each, or a total of 17.5 cysts in the negative tubes. There were 27.5 cysts in all the calculatable tubes in the series. Substituting these values in the MPN equation gives:

$$MPN = \frac{(2)\ (5000)}{2\sqrt{(17.5)\ (27.5)}} = 450.0$$

This number is interpreted to mean that there were 450 viable cysts, most probably, in a suspension of 5000. Or, there was a 9% viability.

d. Normal viability of cysts from routine passage. In using the hemacytometer for making counts of cyst suspensions, only those cysts were counted which were refractile and which did not show coarse granulations nor crenated rims. However, all of the cysts of acceptable appearance were not viable, i.e., all were not capable of excysting and establishing a of trophozoites under the conditions presented to them for this purpose. Among the reasons for this is the fact that all cysts are not quadrinucleate as derived from culture. Harvests were made at approximately seventy-two hours from the time of transfer and at this time it was found that a usefully high proportion of ultimately viable cysts could be obtained. From these harvests, the number of quadrinucleate cysts appeared to fluctuate from 30 to 80%. Cyst production under normal conditions

was not synchronous; some were being constantly formed. It was noted, in the NRS strain, that seventy-two hours did not always coincide with a time when all the rice starch in the sediment had disappeared. However, the period of seventy-two hours for harvesting was adopted, for convenience. It was finally noticed that, if the amount of rice starch originally added was small enough to be completely consumed by seventy-two hours, the cyst yield tended to be higher.

In effect, the percentage of truly viable cysts in each experiment was unknown at the beginning of the experiment and could be expected to be different from the percentage viability of related experiments. It was necessary, therefore, to run controls with each experiment to determine the normal viability of each individual harvest of cysts. The MPN value for irradiated cysts was then referred to the MPN value for the controls, rather than to the original count as visualized.

Twenty-eight sets of control data were selected in order to determine the mean value for rate of cyst viability. The MPN value for each test was adjusted to a percentage value of the original visualized count (see Table 3). It should be stressed that these controls had had no irradiation and had suffered only the amount of manipulation necessary for washing, trophozoite destruction, and quantita-

tive dilution.

The mean for the percentages was calculated by taking the mean of the antilogarithms of the numbers. In this way, a mean of 9.1% was calculated, with a standard deviation of 3.8%. (O). This value for sigma is large because the spread among the values was large, running from 2.4% to 30.2% viability. However, the standard error of the mean was 0.74%, indicating that, if the twenty-eight tests were to be carried out again, under the same conditions, the investigator could be 95% confident of obtaining a mean percentage viability no greater than the value of two standard errors each way, or from 8.6 to 10.6%.

The large spread for values between individual tests reflected inevitable small variations in time of harvest and of handling, but the twenty-eight values show a linear distribution when plotted on logarithmic probability paper. (Figure 8).

This means that the control values for cyst viability are normally distributed in accordance with the theory of probability. It may be assumed, then, that control conditions were indeed similar under which experiments were made and that the MPN measurements were reasonably precise.

e. Experimental irradiation of strain NRS cysts. Cysts, prepared as previously described, were exposed

Table 3

Control Cultures: Percentage of Viable Cysts in Inocula

No.	Date/1955	% Viability	No.	Date/1955	% Viability
1	6/28	9.0	15	9/8	13.5
2	6/30	2.6	16	9/13	12.2
3	7/6	30.2	17	9/15	4.3
4	7/8	9.0	18	9/27	8.3
5	7/12	5.7	19	9/27	18.4
6	7/14	2.4	20	10/4	20.2
7	7/19	18.8	21	10/4	22.7
8	7/21	28.0	22	10/13	13.4
9	7/26	17.2	23	10/13	9.0
10	8/2	4.8	24	10/19	6.2
11	8/4	9.1	25	10/20	6.0
12	8/9	6.1	26	10/30	21.5
13	8/30	7.6	27	11/2	4.1
14	9/6	8.9	28	11/3	2.7

to gamma rays for varying time intervals at a distance from the source (3.6 inches) at which ionization was at a rate of about 900 rep per minute. This value represents only an approximation and the researcher was counselled not to attempt experiments employing exposures of less than ten minutes, since validation of the dose for this short period would be difficult.

Exposure times employed were 10, 15, 18, 20, 25, 30 and 60 minutes.

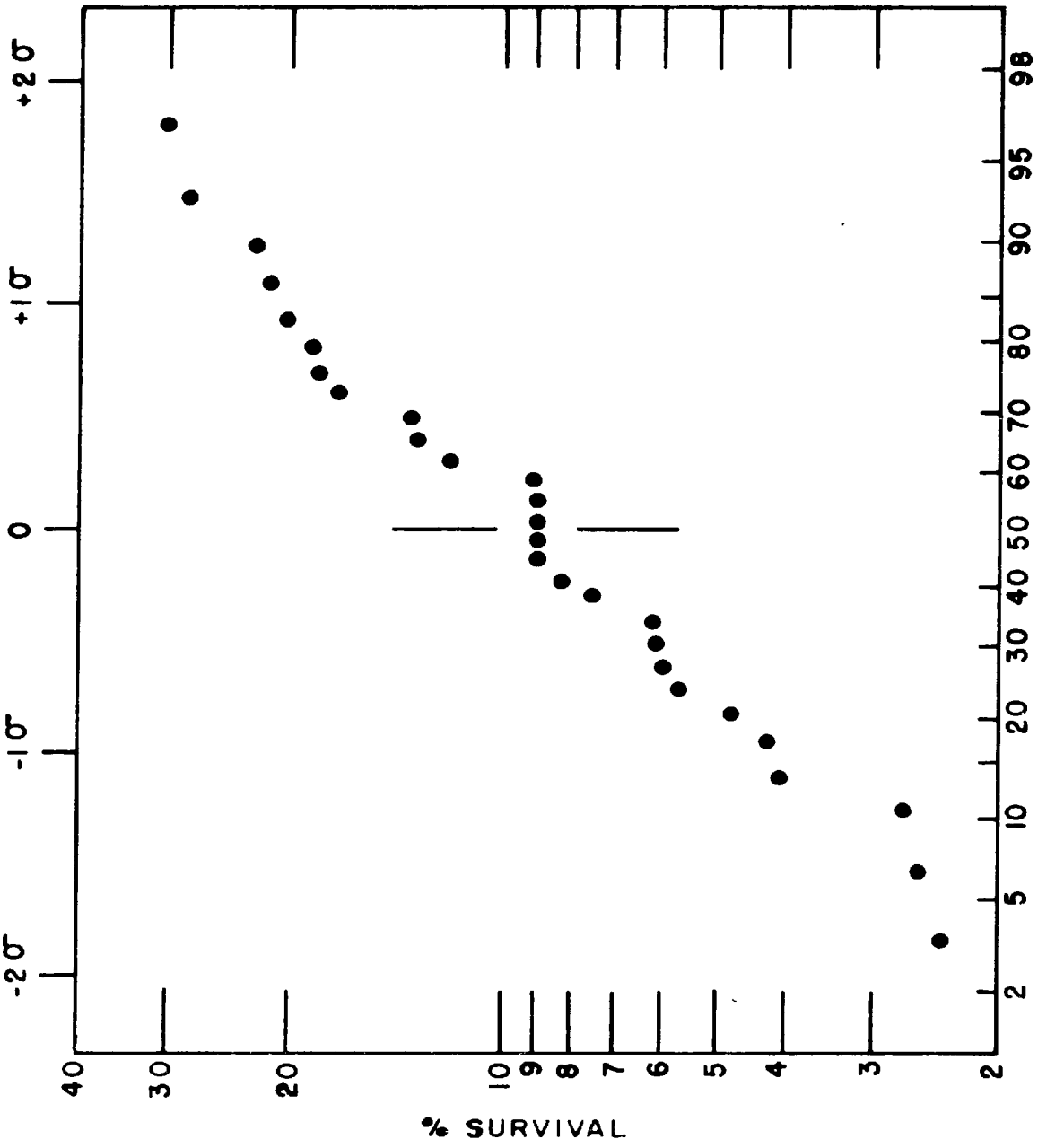
Cysts were concentrated for use at a level of approximately 5,000/ml. Actual counts ran from 4,600 to 7,700 per ml. This variation made little difference in the calculations of MPN values.

A control suspension was prepared with every test suspension. These were placed in the radiation cave, at the same temperature as that at the source, but shielded from the source by a cement wall. Radiation here was hardly distinguishable from the background and was considered negligible.

MPN values were calculated for controls and for irradiated suspensions, using as a value for the "total number of cysts in the suspension" the visualized count as it was obtained from the hemacytometer. The ratio of the test MPN to its control MPN was then determined and expressed as a <u>percentage of viability following irradiation</u>. A list of these

FIGURE 8
VIABILITY OF CYSTS

(LOGARITHMIC PROBABILITY PAPER)



data is presented as Table 4.

Sixteen tests were made, five tests at 10 minutes of exposure each and seven tests at 15 minutes. Two tests at 18 minutes and two at 20 minutes were considered to contribute less reliable values.

Means were taken of the values receiving identical doses and were plotted on semi-logarithmic paper (Figure 9).

The scarcity of points did not warrant making an attempt at fitting a line, although a linear function is indicated by their grouping. However, the positions of the values do not contradict the hypothesis that the destruction of cysts by gamma rays is, like that of trophozoites, an exponential function of dosage, the reaction being of the first order.

f. Effect of increased turbidity on apparent radiosensitivity of cysts. The values for radiosensitivity reported previously are subject to interpretation according to several criteria. One of these, the release of ions from the walls of Lusteroid tubes, has been mentioned elsewhere as a standard condition of all experiments. A second, and possibly more important factor relates to the fact that all organic matter can absorb quanta (Lea, 1955), hence, turbidity of an organic nature in the test suspensions could promote significantly

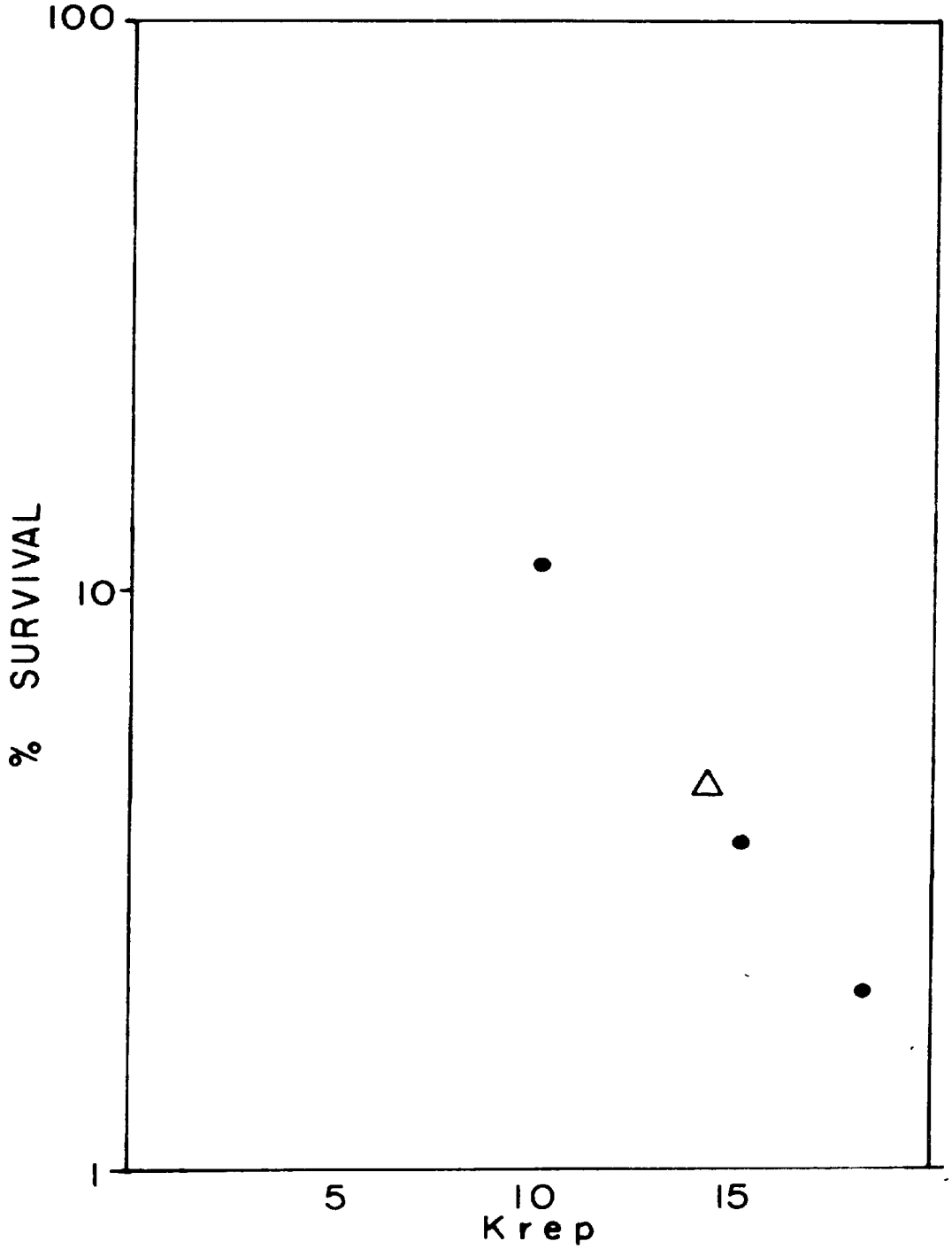
Table 4

Percentage Viability of Irradiated Cysts, Relative to Controls

Test #	Time in minutes	Dosage in rep	% Viability
1.	10	9,000	20.0
2.	"	"	14.2
3.	"	"	11.7
4.	"	"	9.8
5.	"	"	5.7
6.	15	13,200	19.8
7.	"	"	12.4
8.	"	"	6.5
9.	"	"	6.2
10.	"	"	5.4
11.	"	"	3.6
12.	"	"	0.2
13.	18	16,200	2.0
14.	"	"	2.1
15.	20	18,000	1.1
16.	"	"	0.22
	(Unreliable Data)		
17.	25	22,500	0.17
18.	"	"	0.06
19.	28	25,200	0.14
20.	30	27,000	0.0
21.	"	"	0.0
22.	"	"	0.0
23.	"	"	0.0
24.	"	"	0.0
25.	60	54,000	0.0
26.	"	"	0.0

FIGURE 9

PERCENTAGE SURVIVAL OF IRRADIATED CYSTS RELATIVE TO CONTROLS



△ = Center of gravity

heightened resistance values for the cysts.

It was not possible to separate the amebic cysts from all other organic material. Unhydrolyzed rice starch, bits of partially digested egg, and clumps of bacteria are always present. However, according to the usual procedures employed, the amebic suspensions (whether cysts or trophozoites) could usually be called clear by visual inspection.

In order to ascertain the relative magnitude of the protective action of organic turbidity during irradiation, an experiment was devised in which two cyst suspensions, identical except for the nature of the suspending fluid, were irradiated with identical dosages and allowed to grow under similar conditions.

The standard cyst suspension was prepared by pooling the entire contents of eight 48-hour cultures of NRS amebae, centrifuging at 1000-1500 r.p.m. for four minutes, replacing the supernate with sterile distilled water, recentrifuging and rewashing, and storing in the cold room overnight in distilled water. Before using, two aliquots of this suspension were placed in two centrifuge tubes and centrifuged at 1800 r.p.m. for four minutes. In one tube the supernate was replaced by sterile Stone-Locke's fluid. In the other, the supernate was replaced by a turbid fluid consisting of infused egg, bacterial products and bacteria. The preparation of this

fluid was made in the following way. Overlays from eight egg tubes harvested for cysts a week previous to the present experiment were collected at the time of harvesting, and the pool placed in the cold (at 4°C.) for one week. The fluid was then centrifuged for one-half hour at 2000 r.p.m. to remove dead bacteria and any amebae that might still be present. The likelihood of trophozoites being present after the prolonged cold treatment and the centrifugation was considered slight. The top two-thirds of the supernate was collected and used. This fluid had a deep straw color and was noticeably turbid.

The numbers of amebae in the "turbid" and "clear" tubes were each adjusted to approximately 5000 cysts per ml by diluting with the turbid fluid and with sterile Stone-Locke's solution respectively. The final count in each separate preparation was ascertained by means of six separate counts in the hemacytometer.

As a check on the absence of viable amebae in the turbid diluting fluid, five egg tubes were inoculated with the material, three tubes receiving one ml and two tubes receiving two ml. Rice starch and 0.5 ml horse serum were added to each tube. All five tubes were negative for amebae after six days of incubation.

The "turbid" and "clear" cyst suspensions were

distributed in 2.5 ml amounts in Lusteroid tubes, placed side by side in the Masonite carrier (a "clear" tube alternating with a "turbid" tube) in the 3.6 inch radius position. A single twenty-minute exposure was allowed, equivalent, at 3.6 inches, to an overall dosage of approximately 18,000 rep. The cave temperature during this time was 20°C. Controls were placed in the corridor of the cave.

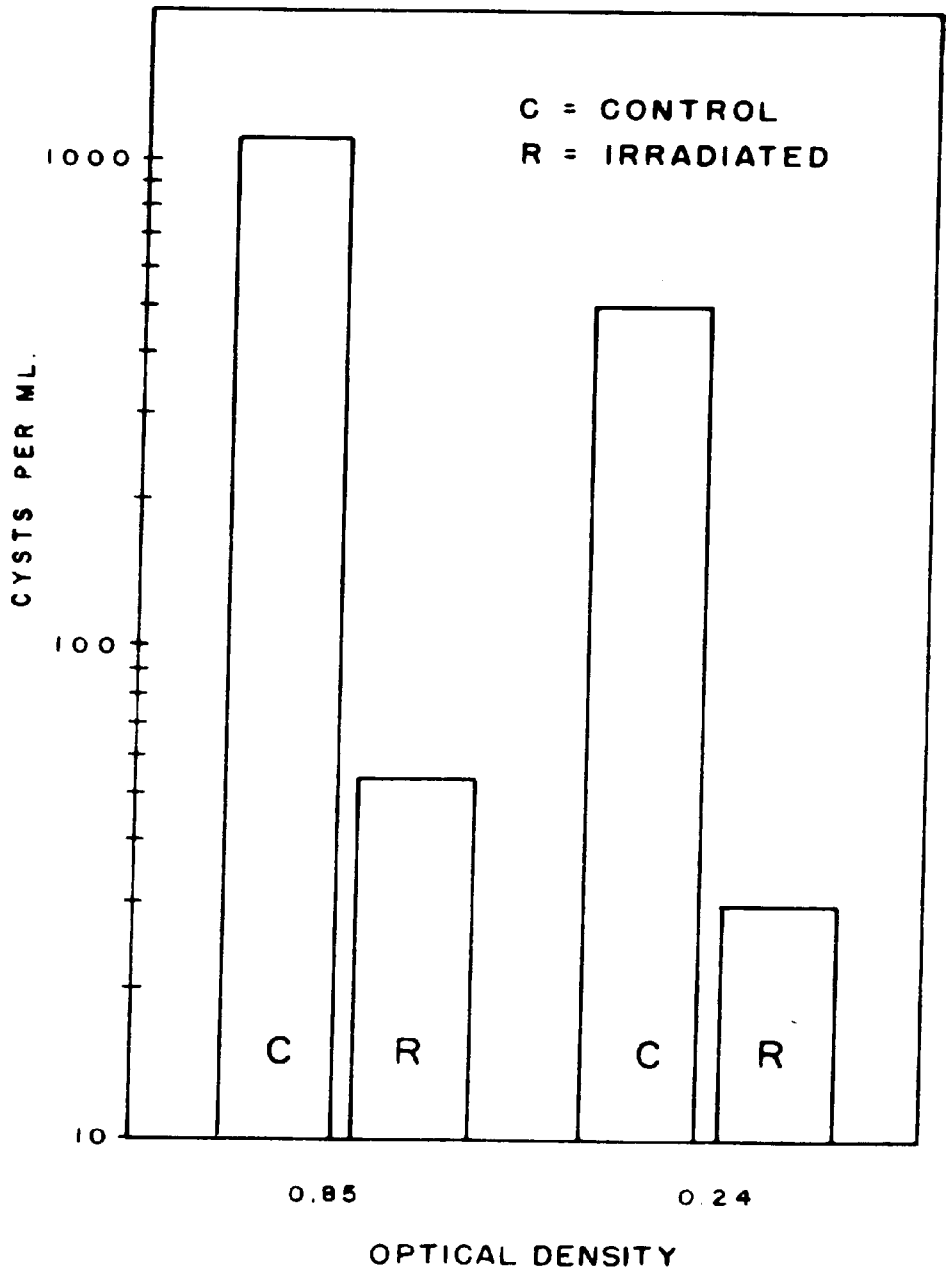
Following exposure the irradiated "turbid" and "clear" suspensions were respectively pooled. For comparative purposes, readings were made on turbidity in the Klett-Summerson photelectric colorimeter, using sterile Stone's Locke solution as a blank.

Figure 10 presents the results of an experiment in which the optical densities of the "clear" and "turbid" suspensions were 0.24 and 0.85 respectively. The height of the columns reads in MPN values for the suspensions. It will be observed that, whereas the absolute values differ between "clear" and "turbid" suspensions, the rate of inactivation by gamma rays was similar in both sets of tubes.

g. Comparative irradiation of cysts of different ages. Routinely, cysts were harvested and washed at 72 hours of growth and were placed in the cold overnight. This was done to insure destruction of all trophozoites. Cysts were always used on the first

FIGURE 10
EFFECT OF RELATIVE TURBIDITY ON CYST DESTRUCTION WITH GAMMA RAYS

(DOSE = 18 KREP)



day after harvesting.

It seemed possible that the cold temperature might have an effect on the radiation response of the cysts. Also, the "age" of the cysts, figures from the time of harvest, could be a factor which might alter the response picture.

To gain some insight into these possibilities, cysts were irradiated on the same day that they were harvested and also on the following day after a night in the cold. The respective "ages" of these cysts, figuring from the time of harvest to the time of gamma ray exposure, were 4 hours and 28 hours. The former are referred to as "young" cysts; the latter are "old" cysts.

Table 5 presents the quantitative information pertaining to three sets of experiments in which different doses of gamma rays were employed. Despite slight differences in quantities such as the density of cysts in test suspensions, the length of time for preconditioning the growth tubes, and the temperature of the radiation cave, it was evident that the main difference between the suspensions used on succeeding days was the age of the cysts and that any consistent difference noted in response could be ascribed to "age."

As in previous experiments, the ratio of test MPN's to control MPN's was expressed as the percentage

Table 5

Irradiation of "Young" and "Old" Cysts

No.	Age	Dose in rep	Inoculum (cysts/ml)	MPN	% Survival
1	Test-Young	9,000	4,900	20	9.8
	Control-Young	-	4,900	202	
	Test-Old	9,000	4,900	8	5.7
	Control-Old	-	4,900	139	
2	Test-Young	13,500	6,600	8	19.8
	Control-Young	-	6,600	407	
	Test-Old	13,500	7,500	28.6	6.2
	Control-Old	-	7,500	456	
3	Test-Young	16,200	5,900	81	2.01
	Control-Young	-	5,900	4023	
	Test-Old	16,200	6,400	28.6	2.07
	Control-Old	-	6,400	1376	

of survival.

In two sets of experiments (Nos. 1 and 3) the percentage of survival ratios resembled each other within the experiment very closely. In experiment No. 2, the difference between 19.8% survival of "young" cysts and 6.2% survival of "old" cysts may be significant. However, differences this large have been observed from experiments in which the cyst "ages" were the same.

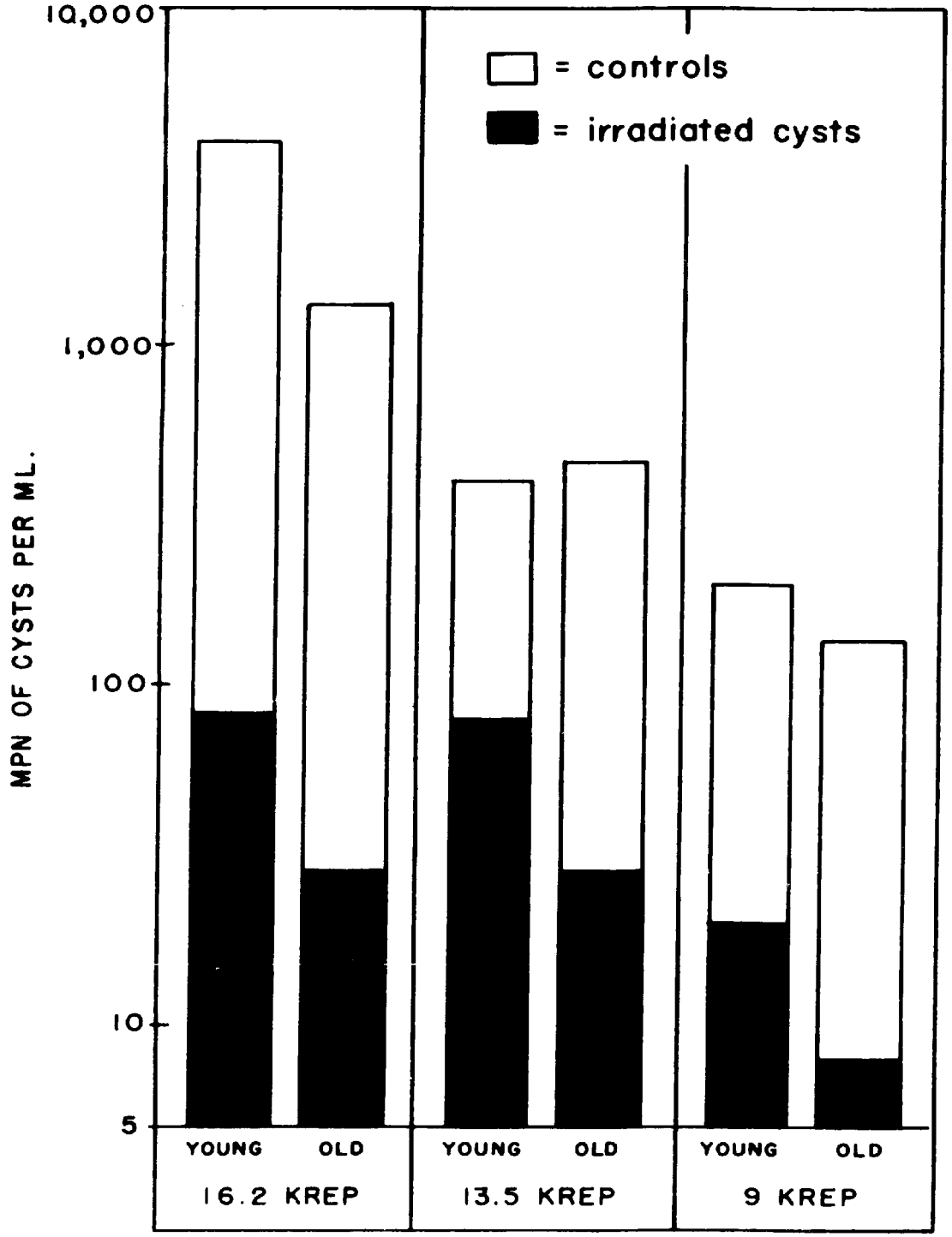
Figure 11 illustrates the fact that the "old" cysts were usually less viable than the "young" cysts. It was a coincidence that the viability of control cysts was progressively less as the gamma ray dosage was lessened. The rate of cyst destruction seemed to be similar always allowing for the initial viability of the unirradiated controls.

It was concluded, therefore, that these data do not tend to support the hypothesis that a difference of 24 hours in the "age" of cysts influences their radiosensitivity.

h. Comparison of Cysts and Trophozoites of Strain NRS. The data indicated that the cysts of E. histolytica were far more radiosensitive than the trophozoites. However, the work with trophozoites and the work with cysts had been performed on organisms of different strains and at different times. It, therefore, seemed advisable to test cysts and

FIGURE 11

EFFECT OF "AGE" ON CYST DESTRUCTION WITH GAMMA RAYS



trophozoites of the same strain by irradiating them together..

A pure cyst suspension was prepared in the usual manner, containing 5,800 cysts per milliliter. It was not possible to obtain a pure suspension of trophozoites, since some cysts are always present in the normal course of NRS growth and cysts are, of course, more resistant to ordinary destructive measures than are trophozoites. Therefore, a suspension of organisms, of which approximately 70% were trophozoites, was obtained from 48-hour growth tubes. These amebae were washed and counted in the manner previously described for trophozoites in Part I, and the suspension was ascertained to contain 46,000 trophozoites per ml.

The trophozoite suspension was divided into two portions. One of these received the same dose as the cyst suspension (32,000 rep). The second portion was irradiated for fifteen minutes (32,000 rep) and then placed in the center well of the source for an additional 40 minutes. At this time (January, 1955) the center well was delivering radiation at the rate of approximately 3,000 rep per minute. Thus, the second trophozoite suspension received an overall dose of 133,500 rep. Controls for all three groups of test suspensions were made by placing similar suspensions in the entrance to the radiation

cave (temperature 4°C.), shielded from the source by a concrete wall.

Following irradiation, appropriate dilutions for performing MPN calculations were made on all the suspensions, after which the organisms were introduced into preconditioned egg tubes. The tubes were examined for a week for the presence of motile amebae.

The results, as shown in Table 6 reveal that the cysts followed a pattern of sensitivity indicated in previous experiments. Reducing the test MPN to a percentage of the control MPN gave a figure of 5.9% survival of viable cysts at 32,000 rep.

In the cyst-trophozoite mixture which received the same radiation as the cysts alone, the ratio of test MPN to control MPN was 74.5%, far larger than that for cysts alone. It should be remembered that the trophozoites only were counted in this suspension and that the cysts were almost one-third as numerous as the trophozoites. But the final dilutions which were made of the trophozoite suspension contained fewer cysts than the similar dilutions of cysts alone.

Finally, the cyst-trophozoite suspension which received more than 133,000 rep revealed a ratio between the test MPN and the control MPN of 0.49%. This level of dosage is almost ten times that which has always proven completely lethal for cysts alone. It could only be concluded, therefore, that the im-

Table 6

Results of Comparing Growth of Irradiated Cysts Alone and Irradiated Cyst-Trophozoite Suspensions

Suspension	Dose in rep	Time	No. organisms per ml in test susp.	MPN	Ratio test MPN/ Control MPN %
Cysts	13,200	15	5,800	81	5.9
Control	0	15	5,800	1397	
Cysts/ Trophozoites	13,200	15	46,000	3018	74.5
Control	0	15	46,000	4047	
Cysts/ Trophozoites	133,500	55	46,000	19.9	0.49
Control	0	55	46,000	4047	

pression gained previously was substantiated by this experiment and that there is a large difference between the radiosensitivity of cysts and that of trophozoites. No claim can be made here for the accuracy of the values of ionizing intensity which were used; the levels of dosage reported represent, as always, approximations. Moreover, there is no evidence that individual variation in sensitivity was absent.

It is realized that the cyst-trophozoite suspensions contained almost ten times as many organisms as the pure cyst suspension. This would not influence the MPN calculations, since the same decimal dilutions were used in inoculating the growth tubes. It may be argued that the presence of more organic material in the trophozoite suspensions acted as a "buffer" against ionization and that this could account for the apparent greater resistance of these forms to the gamma rays. It is probably enough, however, to point out that, if such a "buffer" effect were enough to influence the trophozoites, it should have also protected the cysts. The number of bacteria in all the test suspensions was quite large. But the washing performed in the preparation of the organisms for irradiation produced rather clear suspensions. The large number of trophozoites in the inoculum was used to insure that enough survived

to permit observations to be made (the controls for both experiments involving trophozoites indicated that probably only about nine percent of the organisms survived the manipulations and temperature change involved).

DISCUSSION

I. THE TROPHOZOITE

a. Criteria for Judging Effect. Of the three main kinds of radiation effect usually observed in microorganisms (i.e., lowered division rate, genetic change, and death), the first and last have occupied most of the attention of the protozoologist. An exception is the claim of Kimball (1949, 1955a) that post-autogamous lack of viability in Paramecium reflects genetic alterations in the micronucleus.

The definition of effect varies with authors and is customarily defined in terms of the specific organism under study. It is often difficult to find any effect immediately, especially with medium doses, although the statement of Nikitin (1930) that X rays have no effect on the Protozoa could have been tempered by a more international acquaintance with the literature.

If the organism is relatively large and can be observed individually, (i.e., Paramecium), cessation of motility after a time lapse following radiation may be the criterion (Back and Halberstaedter, 1945). Or, a variation of this will include recording morphological alterations peculiar to the organism, as reported by Koehring (1940) for Chaos chaos. If the organism presents two or more stages, they may

be dealt with separately (as in the work of Feldman-Muhsam and Halberstaedter, 1946, on Leishmania tropica) or together (as in the experiments with malarial sporozoites and trophozoites of Bennison and Coatney, 1945).

Attempts at quantitation differ. Crowther (1926) could count individual paramecia and erect a mortality curve. Halberstaedter (1938) determined levels of trypanosome infection in mice by direct inspection of blood films, using a subjective scale (parasites in every field, very many parasites, field swarming with parasites, etc.).

In the present study, as in most researches of this sort, the amount of information which can be extracted from the data must be modified by the methods for obtaining those data. The prolonged lag phase and the Most Probable Number method compare the number of amebae which actually grow with the number that could grow if the radiation influence were not present. The difference between these numbers must be assumed to represent death of irradiated amebae. But, in order to determine whether the effect of gamma rays on amebae is to produce immediate death or delayed death at mitosis, some criterion other than the two mentioned above must be employed.

It is tempting to compare the results of the present research with the data provided by Back

(1939) who used one-half of the immediate lethal dose of X rays to provoke a lag corresponding to the omission of from four to five divisions in Paramecium caudatum cultures. Recovery, i.e., ultimate division at a normal rate with normal progeny, always took place at such doses. Recovery also occurred with several subcultures strains of irradiated amebae, as described in the present work.

It is unclear, in the studies presented here, whether recovery reflects the individual response or a response of the population. In the sense of the latter, a given dose of radiation is assumed to kill susceptible amebae in a random fashion, according to the "random, discontinuous nature of the rays" (Crowther, 1926). The alternative theory assumes that the death of a single ameba represents an accumulation of insults due to ionization and that division delay with eventual recovery of an individual occurs when the number of such insults is below a maximum. This maximum is subject to individual variation. This is a modification of the "Target" theory, which supposes the presence in the cell of organelles thought to be radiosensitive in different degrees but all essential to the well-being of the organism. The attainment of such an organelle, or "target," by a quantum of energy was considered in the light of statistical probability, with the

likelihood of a hit increasing with time and/or intensity. This was the interpretation given by Holweck and Lacassagne (1930, 1931) for the effect of polonium rays and X rays on the flagellate Polytoma uvella and on some yeasts and molds. These authors attempted to identify the blepharoplast and the centriole of the flagellate with the radiosensitive organelles.

Scott (1937) reviews his reasons for considering the "Target" theory unacceptable (pp. 18-24) and for preferring an explanation of the sigmoid shape of mortality curves based on the distribution of resistance in the population.

The recent trend in the theoretical interpretation of X ray and gamma ray activity seems to be away from the "Target" theory (Curtis, 1951). Biochemical alterations due to ionization have been investigated by several authors. Lawrie and Robertson (1935) reported an initial decrease in ammonia production in Bodo caudatus following 2-4 hours of gamma irradiation, with an increase in ammonia production starting at five hours of exposure and reaching an increase of 42% over the normal after 22 hours. They equated ammonia production with protein metabolism. Kimball and Gaither (1952, 1953) concluded that the so-called "non-genetic" effects of radiation (death and division delay) are enhanced

if paramecia are irradiated in a dilute medium with very few bacteria rather than in a lettuce infusion containing many bacteria. They attributed this largely to the production of hydrogen peroxide outside of the cell. Hypoxia only slightly influenced radiation effects when many bacteria were present, but decreased these effects when few bacteria were present (Kimball and Gaither, 1953). As a "micronuclear mutagen," hydrogen peroxide is ineffective, either alone or in combination with X rays (Kimball, 1955b). It may be mentioned here that, in the present studies, amebic cysts were irradiated in turbid and clear suspensions (turbidity due to bacteria and egg products) and that no evidence of a change in the rate of inactivation by gamma rays was found.

On the other hand, Barron et al. (1949) have advanced evidence that peroxide cannot be of much importance in the inhibition of respiration of Arbacia sperm by irradiated sea water (presumably containing some peroxide) since the sperm suspensions contain a great deal of catalase.

The probability that some of the biological effects of radiation stem from the intracellular oxidation of thiol compounds has been offered by Barron and Flood (1950). When thiol solutions were irradiated in the presence of dissolved oxygen (with consequent peroxide production) the addition of

crystalline catalase gave 17-27% protection against oxidation. Soluble thiol compounds are essential for the process of cell division (Rapkine, 1931). And the inhibiting effects on division of Arbacia eggs by X rays, even at low enough levels to produce an increase in respiration, has been compared to similar effects obtained with thiol reagents and attributed to oxidation of thiol compounds in the cell (Barron and Seki, 1952).

It is interesting to note that Barron and Flood (1950) found reduced glutathione oxidized by gamma rays, although the oxidized form is not reduced. The requirement of E. histolytica for reducing substances in the medium has been reviewed by M. Lwoff (1951).

It is perhaps reasonable to point out that a biochemicsl interpretation of radiation effect is related to the "Target" theory in that certain prosthetic groups attached to molecules, rather than organelles, may be considered as "targets." In amebae, there would be a certain minimum number of radiation-susceptible groups (probably thiol groups) which is the same for both trophozoite and cyst, but which the trophozoite possesses in greater abundance than the cyst. Then, the level of insult required to inactivate the trophozoite will have to be greater (to bring the number of such groups below the minimum"hypothetical" vital) than that for the cyst.

This hypothesis is presented in order to comment on the unexpected difference that was found between cyst and trophozoite sensitivity.

b. Immediate and Delayed Lethal Doses. The range of the direct lethal dose for many Protozoa is usually of a similar high order of magnitude (100,000 r to 600,000 r for ciliates and some rhizopods). The dose required to delay death until the time of the first subsequent division, however, varies but establishes itself in a range roughly one tenth of the value of the immediate lethal dose. Thus, in Trypanosoma gambiense the indirect (or delayed) lethal dose is 12,000 r and organisms which have received such a level of irradiation are unable to establish an infection in an appropriate animal even though they remain motile and apparently unharmed by the exposure (Halberstaedter, 1938). Individual organisms, however, may vary considerably with regard to the delayed lethal dose. Single colonies of the green alga Pandorina morum presented ranges from 3,000 r to 300,000 r (Halberstaedter and Back, 1942).

The physiological effect of doses sufficient to produce death immediately has been thought to differ radically from that of doses which delay death until mitosis. There is evidence that the nucleus and cytoplasm have different susceptibilities. Marshak and Bollman (1936) found that the absorption

of X rays by Urechis spermatozoa was considerably higher than the absorption by substances with little or no nuclear material (erythrocytes, brain tissue, and plasma). Ralston (1939) analyzed the survival curves of populations of the green flagellate Dunaliella salina following X ray exposure and proposed that two separate processes were involved; one, following higher doses, affected the cytoplasm and caused immediate death; and the other, following relatively lower doses, acted directly upon the nucleus in such a way as to inhibit reproduction (killing after a time lapse). Ralston's data indicate that Dunaliella is more than usually radiosensitive for a flagellate, the immediate lethal dose being something more than 18,000 r.

It was concluded by Scott (1937) that X and gamma rays of the order of 100,000 r and more destroy all living tissues indiscriminately in a massive physico-chemical alteration of the substance of both cytoplasm and karyoplasm. The action of lower doses (of the order of 1,000 r) was thought to be on the nucleus alone and to injure the mitotic mechanism.

The physiological significance of the immediately lethal dose, then, must be considered to be small, at least in the Protozoa. On the other hand, the nuclear damage implied by the indirectly lethal dose

invites closer study. Other mechanisms than mitosis may be affected. Thus, exposure of chicken erythrocytes parasitized by Plasmodium gallinaceum to 30,000 r of X rays prevented increase of the parasited upon inoculation into new chickens, but increased appreciably their ability to consume oxygen and glucose in vitro. Exposure to 10,000 r merely reduced their ability to multiply and had no effect upon their respiration (Ceithaml and Evans, 1946). In a different fashion, indirect lethal doses have the effect, in Paramecium cultures, of slowing down the time of cell division, an effect not noticed following the irradiation of Pandorina morum (Halberstaedter and Back, 1942).

c. Monsters. Morphological changes in protozoans following irradiation have been reported, usually following indirect lethal doses or non-lethal doses. The damage is obviously cytoplasmic, although Markovits (1921) associated macro- and micronuclear changes with "strange, monstrous" forms of Paramecium exposed to gamma rays. Also, morphological changes in the nuclei of Entamoeba histolytica were recorded by Nasset and Kofoid (1927) with very weak doses of gamma rays. The larger size of individual Bodo caudatus achieved after continuous but weak gamma radiation (Robertson, 1932, 1935a, b) must be attributed to functions usually ascribed to the nucleus, but

the phenomenon should probably not be included with a description of monstrous forms.

It is also not clear whether vacuolization of the cytoplasm should be considered as a monstrous aberration. Many early workers and some later ones have noticed this phenomenon, often at levels of radiation which seem to approach the direct lethal dose (Schaudinn, 1899; Roskin, 1929; Koehring, 1940). It was probably evidence of "monstrosity" that the free-living amebae of Zuelzer and Philipp (1925) flowed together, following irradiation, to form syncytial masses.

Monsters were not seen in the present work. Unusually large organisms, resulting from continued growth with retarded fission, occur in Colpidium colpoda (Crowther, 1926) after X ray exposure, and in Bodo caudatus (Robertson, 1932) and Entamoeba histolytica (Nasset and Kofoid, 1928) during prolonged exposure to radium. The doses achieved by Nasset and Kofoid may be assumed to have been relatively weak, since death of populations was not recorded.

Very large amebae were seen once or twice by the present investigator in routine control cultures of E. coli and E. histolytica. These gigantic organisms gave the appearance of being multinucleate; they were not observed among irradiated amebae.

Yorke and Adams (1926a) have described giant, multinucleate amebae as occurring in routine *in vitro* cultures.

II. THE CYST

Protozoan cysts have walls which are usually homogeneous, single- or double-walled, chitinous structures (Kudo, 1947). The cyst wall of *E. histolytica* is hyaline in appearance and highly refractile in transmitted light when fresh and viable. The wall is presumably denser than the cytoplasm within for it permits the organism to resist osmotic change. Yet it is not so dense as to be able to protect the organism against desiccation (Reardon et al., 1952). None of this information offers a ready explanation for the apparent difference in radiosensitivity between cysts and trophozoites of *E. histolytica* and the observation is not unique, Brown et al. (1933) irradiated cultures of the hypotrich *Euplotes taylori* and demonstrated that 464,200 r were required to produce immediate death of trophozoites whereas only 400,900 r ensured that cysts would not excyst. The difference is slight and the significance slighter, since different media were employed for the different stages.

Non-lethal doses of radiation have been said to prolong the time of excystment. Taylor et al. (1936) used a single dose of soft X rays (38,400 r) on

cysts of Colpoda steini, recording that the irradiated cysts had a median excystment time of 420 minutes, while controls excysted in a median time of 143 minutes. Using ultraviolet, Giese (1941) found that small dosages of short wave length (around 2654 A) retarded the excystment time of Colpoda duodenaria, whereas longer wave lengths had little of this effect.

It must be remembered that, in the present work, excystment time was not investigated. The Most Probable Number calculation indicates only percentage viability.

One explanation for the present data is, of course, that they are spurious, since the real numbers of organisms irradiated (in the simultaneous run) were quite different in the cyst and trophozoite suspensions. This is a serious objection and one which warrants further experimentation under more closely controlled conditions. Evidence that density of amebae can influence their radiosensitivity has been presented by Stoll et al. (1945), who found that cysts of E. histolytica exposed for ten minutes to 2537 A of ultraviolet were all killed when the suspensions contained 30,000 cysts per liter (30 cysts per ml). When heavier suspensions and shorter exposures were employed, some cysts survived to hatch out but the resulting trophozoites were weak and sluggish compared with controls. On the other

hand, it is unwise to compare data from ultraviolet experiments with those from gamma ray experiments. The increase in penetrability of gamma rays over ultraviolet irradiation is enormous. For example, in cyst irradiations with gamma rays, increased density of the suspending fluid due to bacterial and organic turbidity did not noticeably affect the rate of inactivation. The writer has acquired the conviction, then, that the difference in radiosensitivity between cysts and trophozoites of E. histolytica is real. However, the order of magnitude reported here (the cysts being about 10% as resistant as the trophozoites) will not be insisted upon as absolutely accurate until further work supports it.

The reason for the difference in sensitivity between cysts and trophozoites can be approached only by speculation. It is unlikely that destruction of only one of the four nuclei in a cyst would affect its ability to hatch and thus that the sensitive target area in a cyst is four times greater than in the trophozoite.

A more plausible hypothesis, and one which would permit experimentation, involves the fact that permeability through the cyst wall is lower than through the pellicle of the trophozoite. It is very likely that injurious substances are formed in the organism during irradiations and that the trophozoite

can expel these with greater facility than can the cyst, due to the former's more permeable covering and/or more active metabolic state. Such an injurious agent might be represented by hydrogen peroxide; there is presumably no catalase content in the anaerobic amebae.

SUMMARY AND CONCLUSIONS

1. The normal course of *in vitro* growth of the UC strain of *Entamoeba histolytica* is described. This includes an initial lag phase, a period of logarithmic increase in numbers with one or two peaks, and a sudden or gradual decline.

2. Under the conditions of the experiments, the variability of amount of rice starch introduced in culture tubes did not noticeably affect the length of the lag phase. Population decline was protracted in cultures when the amount of rice starch was increased.

3. Cultures of trophozoites of the UC strain began to display a noticeable increase in lag phase following exposure of inocula to approximately 100,000 rep of pure gamma rays. A dose of 200,000 rep or more resulted in death as indicated by failure of the cultures to grow. The relation between increasing lag phase and increasing doses of gamma rays is represented by a logarithmic straight line.

4. Irradiation did not cause a significant change in the size of amebae.

5. Irradiation did not increase the number of amebae which took the eosin stain.

6. Eventual recovery of the normal lag phase was shown in the progeny of irradiated trophozoites.

7. The viability of normal cysts from routine

cultures of strain NRS amebae was ascertained to be about 10%.

8. A dose of approximately 10,000 rep noticeably lowered the number of viable cysts in a suspension, as calculated by the Most Probable Number method. A dose of approximately 18,000 rep destroyed almost 99% of viable cysts. The relation between increasing cyst mortality and increasing doses of gamma rays is represented by a logarithmic straight line.

9. Increasing the turbidity of the cyst suspension by means of extraneous organic material did not noticeably alter the rate of cyst inactivation by gamma rays.

10. The rate of inactivation was similar for cysts irradiated a few hours after harvesting and for cysts irradiated after overnight storage in the cold.

11. Data are presented which suggest that cysts of strain NRS are roughly one-tenth as resistant to gamma rays as trophozoites of the same strain.

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