X-RAY IRRADIATION OF *COCCIDIOIDES IMMITIS* ARTHROSPORES: SURVIVAL CURVES AND AVIRULENT MUTANTS ISOLATED[1]

J. M. FOLEY, R. J. BERMAN, AND C. E. SMITH

The Naval Biological Laboratory, School of Public Health, University of California, Berkeley, California

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There are 2 morphologically distinct phases in the life history of the pathogenic fungus, *Coccidioides immitis*. The *in vivo* phase consists of spherules which resemble phycomycetous sporangia in that they form endospores by the process of progressive cleavage, and the *in vitro* phase consists of a septate mycelium which produces spores as the culture ages. These spores have been designated conidia (Emmons, 1942) and oidia (Baker *et al.*, 1943) but are generally referred to in the literature today as arthrospores (Smith, 1955; O'Hern and Henry, 1956). They have been variously reported to contain 1 or 2 nuclei (Baker *et al.*, 1943), and to be multinucleate (O'Hern, 1956). The ploidy of the nuclei of neither stage is known.

The nutritional needs of the saprophytic phase are easily satisfied. No vitamins, amino acids, or special growth factors are required, and growth occurs on a wide variety of carbon and nitrogen sources (Baker and Smith, 1942).

Although natural isolates of low virulence are known (Smith, 1951–1952; Pappagianis, 1955; Friedman *et al.*, 1956*b*; Friedman and Smith, 1957), there have been no reports of artificially induced avirulent mutants. Two such mutants, both of which require riboflavin for growth, have been isolated following irradiation of arthrospores with X-rays. This communication describes the altered characteristics of these strains.

A knowledge of the X-ray survival characteristics of arthrospores was a necessary prerequisite to mutation studies with *C. immitis*. Since no studies of this sort have been reported in the literature, a series of survival curve determinations was carried out.

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MATERIALS AND METHODS

Organism. Silveira, a strain of *Coccidioides immitis* highly virulent for mice (Friedman *et al.*, 1956*b*) was used throughout this work.

Cultural conditions and media. Cultures were incubated at 34 C. Stock culture slants were maintained under mineral oil at 4 C on 2 per cent glucose-1 per cent yeast extract (Difco) solidified with 2 per cent agar (Friedman *et al.*, 1956b; modified). Roessler's "natural" agar (Roessler *et al.*, 1946) was used when making viable counts of spore suspensions for animal inoculation.

Solid minimal and complete media which were used to detect auxotrophs were modifications of chemically defined media described by Roessler *et al.* (1946) and Goldschmidt and Taylor (1958). Sorbose was added to these media to restrict colony diameter (Tatum *et al.*, 1949). Their compositions are shown in table 1.

Arthrospore suspensions. Suspensions of arthrospores which retain practically 100 per cent viability for periods of 6 months or longer (Friedman *et al.*, 1956*a*) were prepared by procedures similar to those described by Friedman *et al.* (1956*b*) and by Pappagianis (1955). All spore suspensions were stored at 4 C.

Nuclear stains. Nuclear stains using Azure A were performed at the time of harvest on samples of spore crops employed in irradiation studies, and the ratios of uninucleate to multinucleate spores were determined. A modification of staining methods used by DeLamater (1951) and Huebschman (1952) was used.

Determination of X-ray survival curves. For irradiation, arthrospore suspensions were diluted to a concentration of approximately 1×10^6 per ml and hand-shaken with glass beads for 1 min to produce suspensions containing 95 to 99 per cent single cells and a few chains of 2 or more cells. Samples of a given suspension were pipetted into sterile Lusteroid centrifuge tubes

TABLE 1

Composition of defined media for growth of Coccidioides immitis

Ingredients		Medium[a]	
Compound	Molarity or Amount per L	Minimal	Complete
	M		
KH_2PO_4	0.015	+	+
Citric acid	0.030	+	+
K_2HPO_4	0.015	+	+
$MgSO_4$	0.008	+	+
$CaCl_2$	5.6×10^{-6}	+	+
$ZnSO_4$	6.2×10^{-5}	+	+
$FeCl_3$	5.4×10^{-6}	+	+
$MnSO_4$	0.002	+	+
	mg		
Tyrosine	40	−	+
Adenine	10	−	+
Guanine	10	−	+
Hypoxanthine	20	−	+
Amino acid solution[b]	25	−	+
Pyrimidine solution[c]	10	−	+
Vitamin solution[d]	10	−	+
	M		
NH_4OH	(approx) 0.090	+	+
Glucose[e]	0.011	+	+
Sorbose[e]	0.056	+	+
	g		
Special agar (Noble, Difco)	20.0	+	+

The media were prepared by mixing stock solutions of salts which were 10 or 100 times the desired concentration. The ingredients were added in the order in which they are listed. NH_4OH was added to bring the pH to 6.5. The sugars and the vitamins were sterilized separately and added when the media were poured.

[a] + = ingredient added; − = ingredient omitted.

[b] Amino acid stock solution: DL-alanine, L-arginine-HCl, DL-aspartic acid, L-cysteine, L-glutamic acid, glycine, DL-histidine-HCl-H_2O, L-hydroxyproline, DL-isoleucine, DL-leucine, L-lysine-HCl, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-tryptophan, DL-valine, 1.6 g/L; DL-threonine, 3.2 g/L. The pH of the solution was adjusted to approximately 6 before the addition of the tryptophan.

[c] Pyrimidine stock solution: cytosine, orotic acid, thymine, and uracil, 1 g/L.

[d] Vitamin stock solution (mg/L): Ca pantothenate, choline chloride, nicotinic acid, 200; folic acid, 0.004; inositol, 400; PABA, pyridoxine-HCl, riboflavin, 50; thiamine, 100; biotin, 10. The solution was sterilized by passage through a sintered glass filter.

[e] Sorbose was added to limit colony diameter only when plate counts were to be made. Otherwise glucose alone was added at a concentration of 0.056 M.

which were than capped with rubber stoppers and sealed with masking tape. The tubes were slanted on a circular wooden carrier within the beam of X-rays produced from the gold target of a 2-mev Van de Graff accelerator and were subjected to approximately 1 kiloroentgen (kr) per min for varying time periods. Samples were obtained at 20, 40, 60, 80, and 100 kr by stopping the irradiation and removing one or more tubes from the carrier. The spores, which tend to settle out of suspension, were resuspended by hand-shaking immediately prior to irradiation, and at the time of removal of each sample tube.

Samples were diluted and plated in complete medium. Triplicate sets of plates were made for each dilution, and counts were made on those sets of plates which contained between 20 and 400 colonies per plate. Whenever two sets of plates fell within this range, both of them were counted and averaged. Colony counts were made after the 3rd day of incubation and the plates were inspected for new colonies at 1- or 2-day intervals until the 9th day, after which time no new colonies were observed.

Mutant screening. Spores from irradiated suspensions which had received 80 to 100 kr were plated in complete medium and incubated. The first 1000 well-isolated colonies which appeared between the 3rd and 8th day after plating were transferred to both minimal and complete media. After an incubation period of 7 days, cultures which failed to grow on minimal, and grew normally on complete medium, were selected for further studies.

Characterization of the mutants. The exact nutritional requirements of the mutants were determined by growing them in liquid minimal medium supplemented with vitamins, amino acids, or purines and pyrimidines. The tubes of media were inoculated with spores or mycelial fragments, slanted to increase surface area for sufficient aeration, incubated, and inspected for growth after 5 days. Specific vitamin requirements

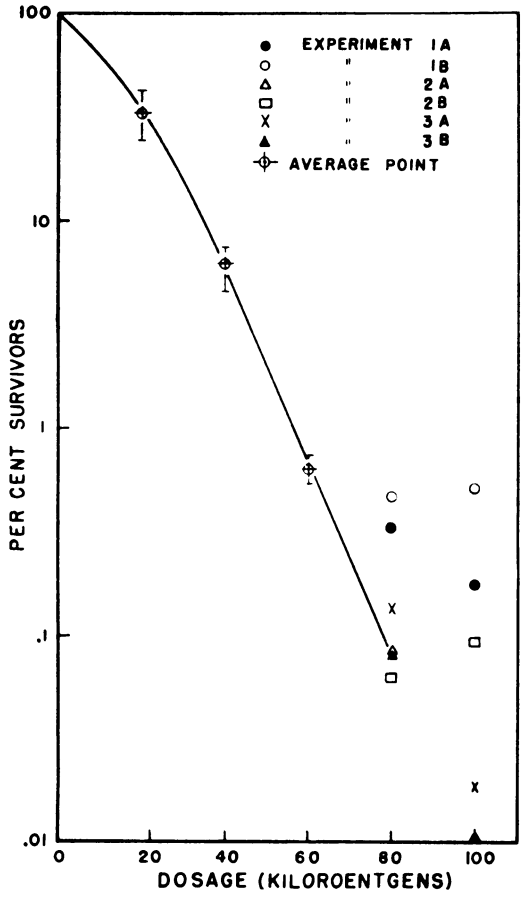


Figure 1. Average X-ray survival curve of arthrospores of *Coccidioides immitis* obtained from 3 separate irradiation experiments. *Experiment 1:* A 13-day-old suspension containing 4.5 × 10^6 spores per ml received 996 roentgens (r) per min. The survivors were counted after 20 (*1A*) and 89 days (*1B*) of storage at 4 C. *Experiment 2:* The same suspension as in experiment 1, 74 days old, 2.3 × 10^6 spores per ml, dosage 945 r per min, counted immediately after irradiation (*2A*) and after 20 days (*2B*). *Experiment 3:* 12-day-old suspension, 3.6 × 10^6 spores per ml, dosage 957 r per min for approximately 30 min, 479 r per min for approximately 20 min, and 957 r per min for remainder of dose, counted immediately (*3A*) and after 20 days (*3B*). The points at 20, 40, and 60 kr are averages of all experiments. The horizontal lines indicate the maximum and minimum counts at each dose.

were determined by similar experiments using single vitamin supplements.

Virulence titrations and challenge to survivors. The virulence of the mutants was determined by the methods of Friedman *et al.* (1955, 1956*b*).

In order to determine whether infection with the mutants conferred immunity, 15 survivors from the virulence tests which had received 1000 spores of the mutants, and a control group of 12-week-old NAMRU mice (Garber and Hauth, 1950; Friedman *et al.*, 1955) were injected intraperitoneally with 117 spores of Silveira each. The animals were then challenged 146 days after the original infection by the intraperitoneal route with a lethal dose of Silveira.

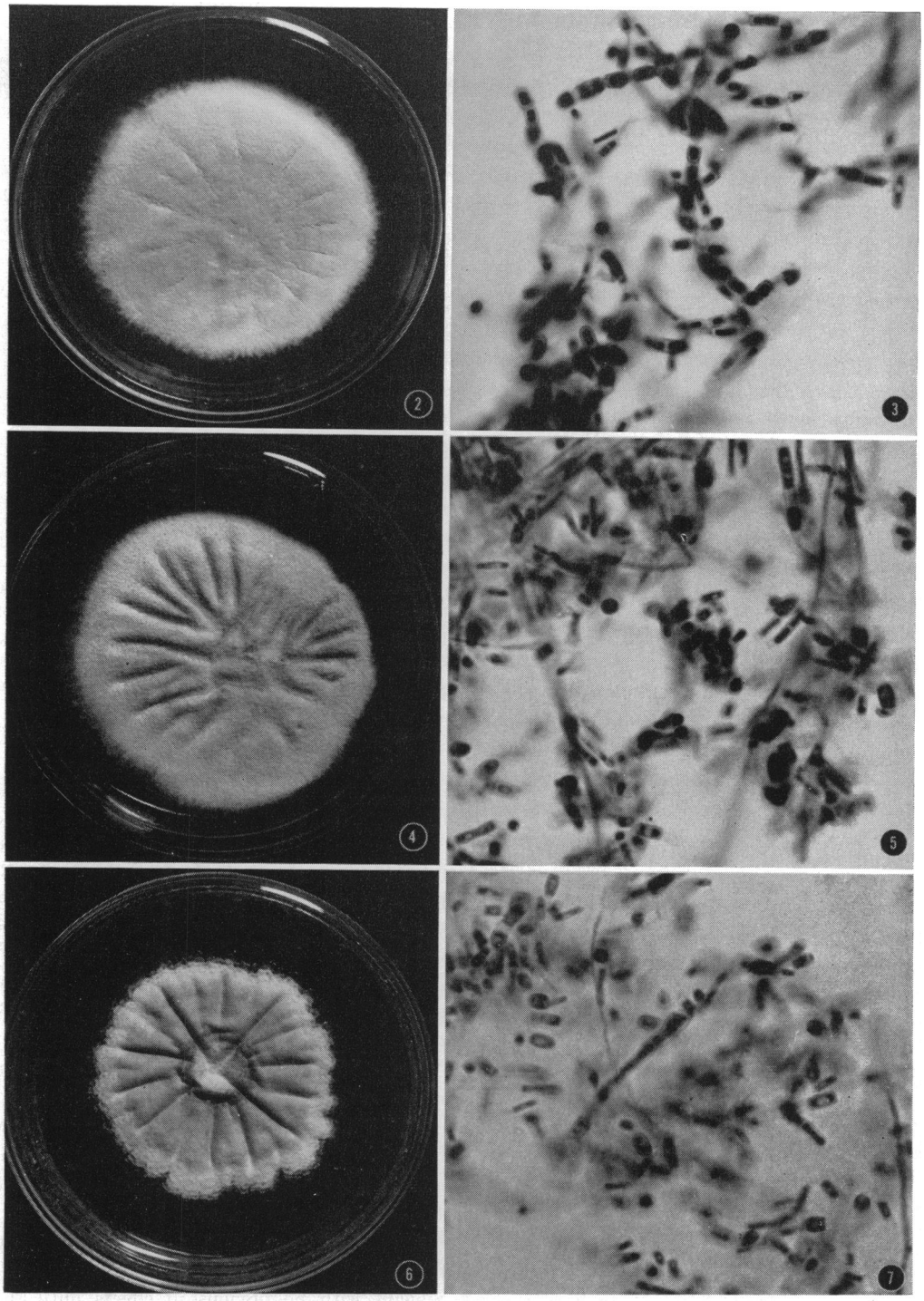
RESULTS

Nuclear counts. Counts on stained spores from the 2 batches which were used in the survival curve determinations (figure 1) revealed a 1:1 ratio of uninucleate to binucleate spores. The actual counts were: suspension 1,516 uninucleate to 484 binucleate; suspension 2,506:495. Only 8 to 10 multinucleate spores were observed in either of these preparations and these were included with the binucleate figures.

X-ray survival curves. The average survival curve obtained after 3 separate irradiation experiments is shown in figure 1. The shape of the curve between 0 and 60 kr is sigmoidal rather than exponential. The results obtained at 80 and 100 kr, as shown, were very erratic. The LD_{50} obtained from this curve is 14 kr.

Auxotrophic mutants isolated. Of the 1000 survivors isolated and screened for ability to grow on minimal medium, there were 49 which had sufficiently striking deviations from the wild type to be retained. Most of these were probably either slow-growing forms, or isolates which reverted to normal very rapidly, since the majority of the 49 eventually achieved some degree of growth on minimal medium. Striking morphological mutants were not abundant. The only one which was noticed and retained was one similar in appearance to a natural variant of strain Venezuela which produces intense darkening of its growth medium (Pappagianis and Kobayashi, 1958). This strain reverted to normal appearance after a few transfers. The only stable and distinctly auxotrophic colonies obtained were 2 isolates, numbers 464 and 887. Both of these were incapable of growth on minimal, but grew normally on complete, medium.

Characteristics of 464 and 887. Both mutants were found to require riboflavin for growth, but they do not appear to be identical, since they have different morphological, growth, and virulence characteristics. Of the 2, strain 464 differs



Figures 2–7. Morphology of wild type *Coccidioides immitis* and of 2 riboflavin-requiring mutants (strains 464 and 887) derived from it. Two-week-old colonies on glucose-yeast extract agar are shown at left, and lactophenol cotton blue mounts of the mycelium (magnification 633×) are shown at right. *Figures 2 and 3.* Wild type, strain Silveira. *Figures 4 and 5.* Strain 887. *Figures 6 and 7.* Strain 464

TABLE 2

Growth of wild type Silveira and of 2 mutant strains in minimal medium supplemented with various concentrations of riboflavin

Conc of Riboflavin	Dry Weight		
	Silveira	Strain 464	Strain 887
mg/L	*mg*	*mg*	*mg*
0.00	57.4[a]	1.4[a]	4.3[a]
0.01	56.0[a]	8.4	4.9
0.05	58.4[a]	26.6	19.4
0.10	83.9[a]	40.5	28.2
0.50	78.3[a]	62.7	31.8
5.00	77.8[a]	51.2	51.8

[a] Weights taken from one flask only. Other values are the average of 2 flasks. Mycelia were grown in glucose-yeast extract broth, washed with physiological saline, and added to flasks of minimal medium plus the concentrations of riboflavin shown. The cultures were shaken for 144 hr, autoclaved, filtered, dried at 50 C over $CaCl_2$ for 7 days, and weighed.

most widely from wild type in morphology. The parent strain grows as a pure white colony on glucose-yeast extract agar and becomes tan and powdery when dried in preparation for harvesting, whereas 464 is gray in color throughout its whole growth period, develops more slowly than Silveira, and is never so powdery in appearance, even when dried. The spores and mycelium appear typical when examined microscopically, but sporulation is greatly reduced. The other mutant stands between Silveira and 464 in all these characteristics (figures 2–7).

An experiment was performed to determine the concentrations of riboflavin required by the mutants (table 2). The results indicate that riboflavin is necessary for the growth of both mutants, and that 887, although closer to normal in all other respects, requires a considerably higher concentration of this supplement than does 464.

Virulence of the mutants. Both 464 and 887 are completely avirulent in the dosage range studied. The results of 2 virulence titrations are shown in table 3. There were no deaths among mice injected with the mutants and more than half the animals were retained for 142 days before termination, whereas the control animals died of coccidioidomycosis in the usual fashion (Friedman *et al.*, 1955). Autopsies of the animals

TABLE 3

Infections and deaths after injection into mice of various concentrations of spores of wild type Coccidioides immitis and of mutants 464 and 887

Strain	Doses (Spores per Animal)	Per Cent Dead at 60 Days	Animals Infected[a]/ Number Autopsied
Silveira (wild type)	10^2	92	46/46
	10^3	96	48/48
	10^4	100	25/25
464	10^2	0	20/25
	10^3	0	16/19
	10^4	0	6/10
887	10^2	0	34/35
	10^3	0	18/19
	10^4	0	24/24

Data for the 10^2 and 10^3 dosages are an average of two separate experiments involving a total of 50 male 14-week-old mice per dose, each injected intraperitoneally with 1 ml of spore suspension. Data for the 10^4 dosage are taken from a single similar experiment involving 25 mice per dose. Actual dosages ranged between 0.9×10^n and 1.2×10^n.

[a] Coccidioidal lesions were observed on autopsy.

injected with the mutants revealed that infection had occurred.

The distribution of the lesions in the animals infected with the mutants was similar to the pathological picture of animals receiving sublethal doses of the relatively avirulent strain 46 (Pappagianis, 1955; Friedman and Smith, 1957). Lesions were small (1 to 2 mm) and confined mainly to the left caudal lobe of the liver, and to the diaphragm.

A further difference between 464 and 887 was noted in these autopsy studies. The infection caused by 887 was generally more extensive than that caused by 464, and more of the autopsied mice showed visible signs of infection than with 464.

Results of the challenge. All of the challenged mice survived the injection of a lethal dose of Silveira with no obvious ill effects until they were disposed of 80 days after challenge. The controls succumbed as expected with a 92 per cent mortality in the same time period.

DISCUSSION

It is not known at present whether the 1:1 ratio of uninucleate to binucleate spores found after staining with Azure A has any significance in interpreting the life cycle of Coccidioides. It is startling that such an exact ratio was found. Since multinucleate spores have been reported (O'Hern, 1956) the condition described may simply be a function of the environment of the spores before harvest. With Neurospora, the number of nuclei per conidium varies with the cultural conditions (Huebschman, 1952), and a more thorough study with Coccidioides might indicate a similar variation.

The fact that the arthrospores which were irradiated were not all uninucleate helps to explain why the survival curves obtained are sigmoid rather than exponential. Studies with microorganisms, especially *Saccharomyces cerevisiae* and Neurospora (Pomper and Atwood, 1955; Norman, 1954) have indicated that an exponential curve is obtained when haploid, uninucleate populations are irradiated, whereas a sigmoid one is obtained when either the ploidy or the number of nuclei is greater than 1 per cell.

Atwood and Norman (1949) have discussed the interpretation of sigmoid survival curves on the basis of a single-hit-per-unit hypothesis. According to this hypothesis, the straight line portion of survival curves such as the one shown in figure 1 can be extrapolated back to zero dosage to give a value, n, which is the average value of the number of units (chromosome sets, nuclei) in the cells of an irradiated population. Thus a uninucleate diploid or a binucleate haploid population of cells would give a sigmoidal curve which, on a semilog plot would have a straight line portion that would extrapolate back to 2.

On the basis of this theory, the curves for a population similar to the irradiated *C. immitis* spore suspensions, which are known to contain uninucleate and binucleate spores in a 1:1 ratio, might extrapolate back to either 1.5 or 3, depending on the ploidy of the nuclei. Theoretically, $n = 1.5$, which would be expected if the nuclei of the arthrospores were haploid, would seem to be the likely number, since, with only a few exceptions, the nuclei of fungi are haploid throughout all but a very short stage in the life cycle. The curves in figure 1, however, do not give an n of 1.5, but rather an n of approximately 3. Although this fact may be due to an entirely unknown phenomenon, it does at least allow for the possibility that the nuclei of the saprophytic stage of *C. immitis* are diploid.

Another possible explanation is that nuclear division had taken place between the time of harvest when the spores were stained, and the time of irradiation. This would seem unlikely since the suspensions were stored under conditions of temperature and nutrition where no growth would be expected to occur, and where growth as manifested by an increase in spore numbers never does occur.

At present it is not possible to give an explanation for the erratic results obtained at 80 and 100 kr. Note that the curves for experiments 1A, 1B, and 2B either form a plateau or actually swing up again. Plateaus have been observed in survival curves of *S. cerevisiae* due to the presence of budding cells in the irradiated population (Beam *et al.*, 1954) and with *Escherichia coli* where they are due to the presence of presumed resistant cells (Gunter and Kohn, 1956).

Considering only experiments 1 and 2, the hypothesis might be put forth that the results observed at 80 and 100 kr were due to recovery from irradiation damage during storage, since only suspensions which have been stored before counting show this phenomenon. However, neither curve for experiment 3 (zero time and 20 days) has a plateau, and the conditions of storage were the same in this experiment as in the other two.

The 2 auxotrophic avirulent mutants isolated during these studies may be added to a constantly increasing list of microorganisms which have lost their virulence once they have lost their capacity to synthesize growth factors (Garber, 1956; Boone *et al.*, 1957). It is interesting that, in this case, the mutant 887, which is closest to wild type in morphology and virulence is the one which requires the higher concentration of riboflavin for growth.

The need for a vaccine against coccidioidomycosis has been frequently noted (e. g., Smith *et al.*, 1957), and considerable work has already been done in this field (Smith, 1951-1952; Friedman and Smith, 1956; Friedman, 1957; Pappagianis *et al.*, 1959). As yet, however, the possibility of using a living, nonvirulent strain has not been realized because of the scarcity of suitable naturally occurring strains (Friedman,

1957). Thus the isolation of these riboflavinless mutants reopens and extends the possibilities of using live avirulent strains of *Coccidioides* for the purpose of immunization.

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SUMMARY

X-ray survival curves have been obtained for arthrospores of *Coccidioides immitis*, using suspensions which were shown by nuclear stains to contain uninucleate and binucleate spores in a ratio of 1:1. The curves are sigmoid in shape and the LD_{50} is approximately 14 kiloroentgens. Two auxotrophic avirulent mutants have been isolated from among the survivors of 80 to 100 kiloroentgens of irradiation. The characteristics of these mutants have been described.

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