THE UNIVERSITY OF ALBERTA

THE SENSITIVITIES OF SOME *PENICILLI* AND *ASPERGILLI* TO GAMMA IRRADIATION, AND THE RADIOSENSITIZATION OF *ASPERGILLUS FLAVUS*

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Sensitivities of Some Penicilli and Aspergilli to Gamma Irradiation, and the Radiosensitization of Aspergillus flavus," submitted by Mirza Mohyuddin in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Spores of Penicilli and Aspergilli, responsible for wheat grain spoilage, are highly resistant to the lethal effects of ^{60}Co gamma irradiation. There was some variability in the doses required to reach an LD_{37} level in the different species of these fungi, but they ranged from 31 to 71 krad. Survival curves for all species followed a sigmoidal pattern, indicating that more than one target was involved in inactivation.

A method for assessing the radiosensitivity of mycelium in fungi was developed. It involved the use of spores at a stage when 2-celled germ tubes were produced. At this stage these mycelial units were readily separable as individuals, thus overcoming difficulties encountered with mycelial clumps.

Chemical compounds, containing iodine, viz., NaI, iodized salt, KI, CaI_2, iodoacetamide and iodoacetic acid were invariably the strongest radiosensitizers of spores of A.flavus, and among these compounds, iodoacetic acid showed the maximum radiosensitizing effect. Other chemicals that conferred radiosensitization on spores included: NaCl, NaBr, KCl, KBr, $NaNO_2$, $NaNO_3$ and vitamin K_5. Compounds, containing calcium cations, viz., $CaCl_2$, $CaBr_2$, CaI_2 and $Ca(NO_3)_2$ behaved somewhat differently. At the low dose of 25 krad they afforded protection to the spores against the irradiation damage, but at higher doses, $CaCl_2$ and $Ca(NO_3)_2$ continued to exert a protective effect while $CaBr_2$ and CaI_2 behaved as radiosensitizers. The reaction of mycelium of A.flavus to

irradiation damage in the presence of these chemicals differed in some instances from that of the spores, but generally it was sensitized to a less degree.

Interaction of heat and irradiation treatments on the survival of spores and mycelium was studied, using 5 different temperatures, ranging from 35°C - 55°C, and 5 different doses, ranging from 25 - 125 krad. In most combinations these treatments showed a synergistic rather than simply an additive lethal effect. The extent of the lethal synergism depended considerably on the sequence in which these treatments were applied. Heating at 45°C, 50°C or 55°C, followed immediately by irradiation, was the most effective synergistic combination for spores, while a simultaneous application of heat and irradiation was most effective in reducing the survival of mycelium.

A time interval of 24 or 48 hours between the heat and irradiation treatments resulted in a desensitizing effect on spores and mycelium, thus indicating the existence of a repair mechanism against these types of damage.

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INTRODUCTION

Ionizing radiations, which include x-and γ-rays, β-particles, protons and neutrons, emitted as a result of intra-atomic readjustments, are being used for preservation, sterilization or shelf-life extension of a variety of foods and food products (6, 42, 45, 54, 74, 75, 88). The microbicidal characteristics of these radiations are well known (22, 29, 41, 79, 90). Ionizing radiations are also useful as a fungicidal and fungistatic treatment. The survival behaviour of fungi, following irradiation, appears to be highly complex. Different radiation doses are required to obtain a complete inactivation of different fungi.

Beraha et al (16) reported the gamma radiation dose response of some decay pathogens. They observed that Phytophthora infestans and Phomopsis citri were much more sensitive to radiation than Cladosporium sp., Gloeosporium spp. Rhizopus nigricans, Stemphylium radicina, Alternaria spp. or Diplodia natalensis. Sommer et al (112, 113) reported a relatively greater resistance of R.stolonifer, Cladosporium sp. and Gilbertella persicaria, when compared with Monilinia fructicola, Penicillium expansum or Botrytis cinerea, which are responsible for the decay of prunus fruit. Saravacos et al (95) observed that the lethal doses for some fruit spoilage fungi varied from 250 to 600 krad of γ-irradiation. They described the "lethal" dose as the one above which growth did not occur. Barkai-Golan et al (12) reported that Rhizopus

nigricans was more resistant to gamma radiation than Botrytis cinerea. A dose of 300 krad reduced the growth rate slightly in R.nigricans, but did not affect sporangial formation, while the same dose totally inhibited the growth of B.cinerea in culture. Vegetative cells of the slime mold, Dictyostelium discoideum,,have been reported to be extremely resistant to radiation (36).

The spores and mycelia are two different structures, and they have been found to vary in their radiation sensitivities. Tascher (120) reported that seed-borne pathogens such as Diplodia zeae and Gibberella saubinetti were more resistant to x-rays in dormant, infected seeds than they were in vitro on potato dextrose agar. Schwinghamer (98) estimated that mycelia of a rust fungus, Melampsora lini, were approximately 20 times more sensitive than its urediospores. He also reported that the urediospores of M.lini Puccinia graminis f. sp tritici, P.graminis f. sp. avenae and P.coronata f. sp. avenae became increasingly sensitive to x-rays, γ-rays and neutrons, when their moisture content exceeded 45 percent.

According to Kljajic (67), conidia of Helminthosporium turcicum and Penicillium expansum were less susceptible than mycelium, while the reverse was true for Botrytis cinerea and Fusarium oxysporum f. vasinfectum. In Aspergillus niger, Ascochyta pisi, Alternaria solani and Trichothecium roseum, conidia and hyphae were equally resistant to radiation. Weijer (125) has shown that there

was a rise in conidial radiosensitivity of Neurospora crassa at different time intervals after commencement of incubation on a shaker. He established a correlation between the increase in radiosensitivity and the duplication of genetic material.

Sommer et al (113) reported that spores of Penicillium italicum required γ-irradiation doses of 10 to 40 krad higher for inactivation than comparable populations of mycelial cells. They also stated that a portion of the difference may reflect difficulties in determining accurately the number of cells in the mycelia. Mycelial cells and conidial populations of Penicillium digitatum required almost identical doses. Sommer et al (110) also reported that, in Botrytis cinerea, young mycelia were more resistant than either mature mycelia or sclerotia, when populations of these structures were equated on the basis of dry weight, protein or DNA content. Many other workers (13, 14, 15, 26, 87, 93) have also reported that in a large number of fungi higher dosages of radiation were required to prevent germination of spores than to suppress the growth of mycelium.

Irradiated fungal spores, seeded on a suitable nutrient medium, may show a variety of changes on germination. Fabre (43) exposed spores of Sterigmatocystis nigra and Mucor mucedo to a high radiation field from a radium source. He found that this prevented the formation of germ tubes. Similar inhibition of germ tubes has been reported in Rhizopus nigricans (73), Penicillium sp. (56) and Aspergillus

terreus (136). Schwinghamer (98) investigated the inhibitory effects of x-rays on the urediospores of Puccinia graminis tritici, P. graminis avenae, P.coronata avenae and Melampsora lini. He also found that γ-radiation from a ^{60}Co source produced inactivation similar to that obtained with x-rays. Cathode rays inhibited germination of spores of Rhizopus nigricans (73) and Penicillium sp. (56).

Exposure of Penicillium notatum spores (55) and the urediospores of the 4 rusts, studied by Schwinghamer (98), to either slow or fast neutrons, resulted in the inhibition of germ tube formation. Fast neutrons were more effective in causing this inhibition. Alpha particles from polonium inhibited germination in Aspergillus niger (18, 135) and in A.terreus (136).

Ionizing radiations have been reported to stimulate germination of fungal spores, in contrast to their inhibitory effects on germination. Buchwald and Whelden (23) irradiated spores of Aspergillus niger with low voltage cathode rays (1.5 - 3 K.V.) and found a statistically significant stimulation on germination. Stimulation was evidenced in: (i) earlier signs of swelling, (ii) more rapid swelling, (iii) larger average size at any given time and, (iv) higher percentage germination as compared to non-irradiated spores. According to them, cathode rays may have caused permeability changes in the cell wall, allowing water to enter and thus initiating swelling. They believed that primary sites of energy absorption and ionization were in the spore wall,

cytoplasmic membrane and outer portions of the cytoplasm, because the energies of cathode rays are too low to penetrate deep into spores to cause ionizations in the nucleus. They stated that more likely an osmotic imbalance, caused by large number of ions released within its substrate, was responsible for drawing water through the cell membrane.

Rodenhiser and Maxwell (92) observed that the promycelia from teliospores of Ustilago hordei, that had survived low x-ray doses, were much longer than those of non-irradiated teliospores. Sansome et al (94) reported an acceleration in the germination of Neurospora crassa spores that had survived x-irradiation treatment, when compared to non-irradiated spores. They visualized some changes in the spore membrane which facilitated water uptake and thus hastened the process of germination. A further support to this speculation came from the fact that, at high radiation doses, many spores did not germinate, but they showed swelling. Savulescu and Becerescu (97) reported that a 1000 roentgen x-ray treatment had a stimulatory effect on teliospore germination in some species of the genera, Ustilago and Tilletia.

An increase of up to 25 % in germination of spores of Neurospora crassa, treated with a dose of up to 7 krad of x-and γ-rays, has been reported by Woodward and Clark (132). They also observed that radiation doses between 500 and 50,000 roentgen accelerated the germination process in those spores that survived. Weijer (126, 127) also observed

x-ray stimulation of conidial germination in Neurospora crassa, and suggested that the stimulatory action was superimposed on destructive effects, which became apparent at higher doses. He believed that the stimulatory and lethal effects might represent different degrees of damage to the cell membrane or changes in membrane permeability.

X-rays were reported to have no effect on the germination of spores of Chaetomium cochliodes (39), C. globosum (46), Collybia dryophilia, Fusarium batatatis or Sclerotium bataticola (62). The radiation dose in some of these experiments may have been too low to cause any inhibition of germination because spores of Neurospora tetrasperma (122) and Ustilago hordei (92) were shown to germinate in great numbers after doses of x-rays as high as 100 kr.

The effect of irradiation on mycelium has been investigated and there have been reports of retardation of mycelial growth in cultures of Mortierella (33), Collybia dryophila, Fusarium batatatis and Sclerotium bataticola (62), after exposure to radium sources. Observable reduction in the growth rate of cultures of Phycomyces blakesleeanus has been noted by Forssberg (47) after exposure to low doses of 0.001 roentgen of x-or γ-rays. Similarly, vegetative growth, as well as sporulation, were inhibited in cultures of Chaetomium globosum, Lenzites trabea, Aspergillus niger, A. flavus and a species of Penicillium, exposed to radium and polonium sources (17, 18).

Fabre (43) reported the inhibitory effects of high doses of ionizing radiation on the vegetative growth of fungi. He also reported a stimulatory effect on the growth of mycelium of Mucor mucedo after exposure to radiation. Chavarria and Clark (27) studied the effects of low x-ray doses on several skin pathogenic fungi. They observed a stimulatory effect on growth of colonies. These workers concluded that the curative action of x-rays against skin infection resulted from a stimulation of the defence mechanism of the skin rather than a deleterious effect on the fungi themselves.

A temporary retardation in growth was reported by Beraha et al (14, 15) and Nelson et al (87) in colonies of Rhizopus stolonifer, Penicillium italicum, P.digitatum and Botrytis cinerea, following irradiation at a dose insufficient to inactivate them permanently. Growth was then resumed after a delay of several days. According to these authors, the basis of the effect of this delay is not well understood. It is likely, however, that most of the mycelium had been irreversibly damaged. Normal growth was probably resumed from localized areas from certain portions of the hyphae.

Malla et al (76) noted the in vitro susceptibility of strains of Penicillium viridicatum and Aspergillus flavus to β-irradiation. According to their observations, regardless of the species or strain studied, an increase in the radiation dose decreased the development of conidia and

growth of mycelia.

Various growth processes in fungi show differential radiosensitivities. It has been observed in Chaetomium cochliodes (39), Neurospora tetrasperma (122), Ustilago hordei (92) and Aspergillus terreus (115) that, after x-irradiation, many spores produced germ tubes, but not all of them formed colonies. A great number of these spores died shortly after germination. These results suggested that the germination process was more radiation-resistant than growth processes. Zirkle (135) and Zirkle et al (136) observed that, within the germination process itself, the spores of A.niger and A.terreus were much more resistant to x-rays and α-particles in the swelling phase than in their sprouting phase.

Berk (18) confirmed the results of Zirkle with A. niger. In addition, he showed that the next most resistant process was cell division or vegetative growth, while sporulation was the least resistant process to irradiation with α-particles. Sommer et al (107) also observed that the ability of the spores of Rhizopus stolonifer to swell prior to emergence of the germ tube was a more resistant process than germination.

Beraha et al (14, 15) reported that, in many fungi, the capacity for indefinite growth is lost at a much lower dose than the ability to germinate. In experiments with Rhizopus stolonifer, a dose of 500 krad reduced the surviving fraction (i.e., the ability to form a colony) to less than 1 %,

but hardly affected their ability to germinate. In order to reduce germination to near 1 %, a dose of 1500 krad was required.

Sommer *et al* (108) investigated the response of various fungi to gamma radiation and concluded that survival, as indicated by potential for unlimited growth, was generally more sensitive than the germination process. They also demonstrated that irradiated, abnormally-germinating sporangiospores of *Rhizopus stolonifer*, though incapable of forming a colony, were able to produce pectolytic enzymes almost similar to those produced by non-irradiated spores. Karoly (64), who investigated the effects of 1.2×10^6 rads of x-rays on mycelia of *Aspergillus foetidus* and *A.niger* reported that the fungal cells lost their reproductive power, but the activity of their pectolytic enzymes was not affected.

The multitude of effects of ionizing radiation on fungi, reviewed in previous sections, partly reflects the variation in resistance among different genera and different species. It is difficult to make comparisons because of the many different conditions under which the experiments were carried out. In summary, it appears that high doses of ionizing radiation inhibit spore germination and retard vegetative growth. The effect of low doses are more difficult to evaluate. In some instances ionizing radiation had no effect on the number of spores that produced germ tubes, while, in others, it was shown that many of these

spores are so damaged that development may cease altogether or continue at a reduced rate. Low radiation doses may also stimulate germination, possibly through changes in permeability.

The complexity of the effects of irradiation on fungi emphasizes the importance of selecting a reliable criterion for survival. Ubber and Goddard (122) showed that, in Neurospora tetrasperma, the survival curves of spores, based on the inhibition of germination and on colony-forming ability, were completely different because irradiated spores often germinated but did not develop further. They suggested that, after irradiation, there is no relationship between germination and growth, except that the former is a pre-requisite for the latter.

They also concluded that spore germination is not a valid criterion of the viability of the cell and, therefore, it cannot be used to obtain data to test the quantum hit theory of lethal action. Woodward and Clark (132) found that low doses of x-rays increased the percentage of germination of spores of Neurospora crassa. They suggested that in some cases, the survival curves might be the result of two independent effects: (1) spore "activation" or stimulation, which would prevail at low doses of radiation and, (2) spore inactivation, which would be the dominant effect at high doses. In such cases, a survival curve, based on the criterion of germination ability, might be different from one based on the criterion of colony-forming ability. This

is further supported by the fact that Whelden (129) and Rodenhiser and Maxwell (92) have shown that spores, stimulated to germinate by radiation, often failed to continue development after producing a germ tube. For these reasons the colony-forming ability of an irradiated spore was used as the criterion of its survival in most studies of the kinetics of radiation inactivation.

With an increase of radiation dose, there is a decrease in the number of survivors. There is a relationship between the amount of radiation dose applied and the number of survivors. Survival curves are plotted to express this relationship. The logarithum of the survivors is plotted on the y-axis, and radiation on the x-axis of a graph. The shape of the dose response curve may be either exponential or sigmoidal, depending upon the strain or species studied, the type of irradiation, the irradiation technique, growth phase of the cells and other factors. According to Hutchinson (61), the plot of the survivors of more complex organisms, as a function of dose, is usually sigmoid (if the dose is plotted on a linear paper) with a shoulder at low doses, and an exponential slope at high doses (if survivors are plotted on semilogarithmic paper). This is interpreted as meaning that either multiple targets are present or multiple hits in the same target are needed for inactivation.

Both exponential and sigmoidal survival curves are found for fungal spores subjected to ionizing radiations. These two types of curves may be obtained for spores of the

same species exposed to different types of radiation.

Sigmoidal survival curves were reported for the spores of Rhizopus nigricans, irradiated with cathode rays or x-rays, when the criterion for survival was the colony-forming ability of the spores (73). Similar curves were obtained for the x-ray inactivation of spores of Chaetomium cochliodes (39) and Neurospora tetrasperma (122). Using the ability to form a germ tube as the criterion of survival, it was found that the survival curve for spores of Aspergillus terreus, irradiated with x-rays, was sigmoid, while irradiation of A.terreus and A.niger with α-particles resulted in exponential curves (135, 136). The colony-forming ability of a sample of spores of Streptomyces flaveolus was found to be inactivated in exponential fashion by x-rays (65), although it was observed that the rate of inactivation was slightly greater at lower doses. Savage (96), however, reported that x-irradiated spores of Streptomyces griseus were inactivated in a strictly exponential fashion.

Stapleton et al (115) found a sigmoid relationship between colony-forming ability and x-ray dose in spores of Aspergillus terreus, but exponential curves were obtained when α-particles and protons were used. The colony-forming ability of spores, produced by heterokaryotic strains of Neurospora crassa, was inactivated in exponential manner by x-rays or ^{60}Co. γ-rays (8).

Sigmoid survival curves were obtained for uredio-spores of Melampsora lini, Puccinia graminis tritici, P. graminis avenae and P. Coronata avenae, after irradiation with x-rays, ɣ-rays, neutrons or ultraviolet radiation (98). Similarly, multinucleate spores of Neurospora crassa, irradiated with ^{60}Co ɣ-rays, produced sigmoid survival curves, but a strain producing only uni-nucleate spores was inactivated in an exponential fashion (48). Under the same conditions V. Hofsten (58) found that, in contrast to sigmoid ultraviolet survival curves obtained for Ophiostoma multiannulatum, x-irradiation of this fungus resulted in exponential survival curves. Colony-forming ability was the criterion of survival in both cases.

The target theory of radiobiological action has been of great value in interpreting the significance of survival-curve shapes obtained with various types of ionizing radiation. Although many early workers proposed that the shape of the curve might be a reflection of biological variation in resistance among the irradiated population or the threshold accumulation of radiation-produced toxic chemicals, these explanations were not as satisfactory as later ones based on target theory analysis. Bacq and Alexander (10) discussed the application of this type of analysis to the biological effects of ionizing radiation. These authors have also pointed out the expected results of the interaction of various types of radiations with targets of varying size and distribution within the cell.

Zirkle et al (136) found that spores of Aspergillus terreus yielded exponential survival curves, when irradiated with α-particles, and sigmoid survival curves fitting 2-hit theoretical curves, when x-rays were used. They concluded that the number of entities that must be altered in each cell must be small and that, while all of them are altered by one suitably located α-track, at least two electron tracks are required to produce the same alterations. Stapleton et al (115), using the same organism, obtained the same relationship between the type of radiation and the shape of the survival curve. However, they believed that the x-ray survival-curve shape was closer to the theoretical curve for 3 hits. Furthermore, they found that spores of Aspergillus terreus were inactivated in an exponential manner by protons. The densely ionizing particles were more efficient than x-rays in killing the spores, a fact which was again consistent with the hypothesis that several electrons are required to produce the same quantitative effect as a single particle or proton. These workers suggested that chromosomal damage might be responsible for the lethal effects of ionizing radiations.

According to the original target theory, a single ionization, occurring directly inside a sensitive site within a cell, was responsible for its inactivation. This assumption precluded alteration of the biological effect of ionizing radiation by environmental conditions. However, it was observed that various environmental factors could

influence the effects of ionizing radiations. Stapleton and Hollaender (114) reported that the presence of oxygen increased lethality of x-ray irradiated spores of Aspergillus terreus, and noted also that, as the water content of the spores increased, they became more sensitive. Urediospores of Melampsora lini, Puccinia graminis tritici, P.graminis avenae and P.coronata avenae were also observed to be more sensitive to x-and γ-rays, as their water content increased (98). Similar findings of the enhancing effect of oxygen, increased water content, and other environmental factors in other organisms, were interpreted as indicating that not only sensitive sites within the cell were inactivated by direct ionization, but that ionizations,occurring in the vicinity of the critical sites,were also sufficient to cause their inactivation. Thus, the inactivation of cells may be brought about by a combination of direct and indirect actions.

Gafford (48) found evidence that,when spores of Neurospora crassa were irradiated with γ-rays in aqueous suspensions under aerobic conditions, survival curves indicated the occurrence of two different inactivation processes The more effective one is an indirect process that requires the presence of water and oxygen, and results in nuclear inactivation. The second process is a direct hit process that cannot be definitely associated with any particular cellular component. When the spores are lyophilized and irradiated in vacuum-sealed tubes, only the second process is

effective in killing.

Besides ionizing radiation, heat is a well known fungicidal agent. A considerable amount of information regarding the lethal temperatures for spores of many fungi is available. Much of the literature, describing the effect of temperature on the growth of mycelium, has been reviewed by Wolf and Wolf (131), Hawker (57) and Cochrane (30). Recently, Deverall (38) reviewed various aspects of temperature effects on fungal growth. There is comparatively a dearth of information on the kinetics of heat inactivation in fungi. Quantitative studies, comparing heat sensitivity of spores and vegetative cells, are also lacking. This is partially because such a study is complicated by the filamentous growth of most vegetative cells.

Smith (104) exposed the spores of Botrytis cinerea to temperatures ranging from 31° to 50.3°C in aqueous suspension and, using the ability to germinate on agar plates as the criterion for survival, he obtained a series of sigmoid curves when survival was plotted as a function of heating time at several temperatures. All curves were alike, except for a change in the rate of killing at different temperatures. He showed that the general shape of the curve agreed with a recognized type of frequency distribution and concluded that resistance to heat inactivation was distributed in a similar manner throughout the spore population. Ascospores of Byssochlamys fulva were found to be highly resistant to heat (60). The temperature, usually employed to

destroy most fungi, served to activate the ascospores of this species.

Yanagita and Yamagishi (133) found that short exposures of spores of Aspergillus niger to high temperatures resulted in instantaneous death. Survival curves for spores and mycelial cells, exposed to temperatures between 43°C and 55°C for 3 minutes, were sigmoid and nearly superimposable, except that the spore survival curve was shifted in the direction of the higher temperatures. The temperature required for 50 % killing of the spores (LD_{50}) was 51.5°C, while the corresponding temperature for mycelial cells was 46.5°C. The parallel slopes of the curves suggested that variation of heat sensitivity among spores and vegetative cells was of the same order of magnitude.

Reports, quoted previously, indicate that microorganisms, causing food spoilage, are highly resistant to the lethal effects of ionizing radiations. Thus, the irradiation dose required to eradicate these microbes could render the food unwholesome for human consumption. Various physico-chemical agents, when present during the time of irradiation, result in a sensitization of the microorganisms, thus reducing the radiation dose required for their inactivation. Extensive investigations have been carried out with several chemicals which enhance radiation inactivation of bacteria. There is a possibility that these chemicals can be of some technological importance.

Some of the substances that have been used are

oxygen (4, 59, 123, 124), iodoacetamide, iodoacetic acid and related compounds (19, 32, 34, 35, 69, 82), halogeno-phenols (77, 84), alkali halides (78, 85, 86), vitamin K_5 and its analogs (99, 103) and N-ethylmaleimide (20, 21). Most of these investigations have been conducted on radiation-resistant bacteria. Silverman (101) discussed various aspects of microbial radiosensitization and,recently, de Proost (37) completed a review on radiosensitizers in food irradiation.

Chemical radiosensitization of yeast has been only occasionally reported in the literature (99, 102). Recenlty, Dupuy and Tremeau (40) reported that diethyl-pyrocarbonate sensitizes both budding and non-budding yeasts. They concluded that inhibition of enzymes, through their combination with thiol groups,was responsible for sensiti-zation. Cycloheximide, in combination with gamma irradiation, had a synergistic effect on Saccharomyces cerevisiae (25).

There have been only a few reports on radiosensiti-zation of filamentous fungi with chemicals. Recently, Georgopoulos et al (49, 50) studied the radiosensitizing effect of various chemicals on fruit spoilage molds and yeast, viz., Penicillium italicum, Rhizopus nigricans, Botrytis cinerea, Aspergillus niger, Aureobasidium pull-ulans and Candida tropicalis. The chemicals tested were vitamin K_5, 2 methylnaphthalene, I-naphthol, I-naphthylamine, 4 amino-I-naphthol, 5 amino-I-naphthol, naphthoresorcinol, p-amino-phenol, iodoacetamide, iodoacetic acid, maleic acid

and N-ethylmaleimide. They observed that, in the presence of oxygen, iodoacetamide was a very effective radiosensitizer for all fungi tested. In Botrytis cinerea and Rhizopus nigricans, iodoacetamide induced destruction of spore germinability by very low radiation doses. Iodoacetic acid also sensitized the two species on which it was tested. I-naphthol achieved a considerable increase of radiolethality in Aureobasidium pullulans and Candida tropicalis, but not in four species of typically filamentous fungi.

Barkai-Golan and Kahan (11) investigated the combined action of diphenyl and gamma radiation on the in vitro development of fungi, pathogenic to citrus fruits. The combined action was tested on Penicillium digitatum, P. italicum, Diplodia natalensis, Phytophthora citrophthora, Oospora citri-aurantii, Trichoderma viride, Alternaria citri and Colletotrichum gloeosporioides. According to these workers, the combined action of 5 or 20 mg diphenyl/dish and a sublethal dose of 60 krad of ^{60}Co γ-rays completely inhibited the growth of Penicillium digitatum and Diplodia natalensis, and the combined action of diphenyl and 140 krad inhibited growth of P.italicum. A combined treatment of diphenyl and 60 krad of gamma rays completely inhibited the growth of Trichoderma viride. The combined action of diphenyl and radiation on Phytophthora citrophthora, Oospora citri-aurantii, Colletotrichum gloeosporoides and Alternaria citri had no increased effects over each of these treatments used separately.

Heat, as a sensitizing treatment of

irradiation in bacteria, has been investigated by several workers (53, 63, 66, 71, 72). Stehlik and Kaindl (116) conducted detailed investigations on the combined effect of heat and irradiation on Saccharomyces cerevisiae var. ellipsoideus. A temperature range of 20^{o} - $52.5^{o}C$ was used. They reported that the most effective combination was the simultaneous application of heat and irradiation; less effective was the combination in which irradiation at room temperature followed the heating; and least effective was the heating after irradiation at room temperature.

Sommer et al (111) studied the radiation-heat interaction in Monilinia fructicola, Botrytis cinerea, Penicillium expansum, Cladosporium herbarum and Rhizopus stolonifer. They reported that interaction between treatments sometimes caused a 5-to 10-fold increase in inactivation. The amount of synergism and the preferred sequence of application for maximum fungicidal effect depended upon the pathogen. They observed that, with Rhizopus stolonifer, the maximum effect occurred when irradiation preceded heating. In all other species, the reverse sequence resulted in greatest inactivation. Sommer et al (106) demonstrated that heating sensitizes B.cinerea to irradiation more than irradiation sensitizes it to heating. A partial desensitization of the fungus has been found to be associated with delays between heating and irradiation.

Wheat grains in storage are easily spoiled by microorganisms. According to Christensen and Kaufmann (28),

fungi are the major causal agents for grain spoilage under ordinary, aerobic storage conditions. Mossel and Ingram (81) listed the Aspergillus - flavus - oryzae group as the most important in grain deterioration. This group, according to Thom and Raper (121), is cosmopolitan in its distribution and omnivorous in substrate utilization.

Gamma irradiation in the dose range of 16,000 to 65,000 rep , emitted from Cobalt 60 and Cesium 137, has been successfully used for controlling insect infestation in stored wheat grain (31, 52, 80). There is, however, a scarcity of information regarding the effects of γ-irradiation on wheat grain spoilage fungi. Yen et al (134) reported that a dose of 3,750 krep was required for complete destruction of all the fungi in wheat grain. Such treatment, no doubt, stops the growth of fungi, but the high dosage eliminates seed germination and may result in unwholesome nutritional properties in the grain.

The following aspects of irradiation of grain spoilage fungi were investigated:

(1) Determination of sensitivities of species of Aspergillus and Pencillium to ^{60}Co γ-irradiation.

(2) Radiosensitization of spores and mycelium of Aspergillus flavus by chemicals.

(3) a) Radiation-heat synergism in spores and mycelium of Aspergillus flavus.

b) The effect of time interval between heat and irradiation treatments on the sensitization of spores and mycelium of Aspergillus flavus.

MATERIALS AND METHODS

A. Radiosensitivities of Grain Spoilage Fungi

1) Organisms

Species of Aspergillus and Penicillium, obtained from a collection of Mr.H.A.H. Wallace, Canada Department of Agriculture Research Station, Winnipeg, Manitoba, were used in these studies. These fungi were isolated from stored wheat grains over a period of several years. Their identification was recorded as follows:

Name of the Fungus	Culture No.
Aspergillus versicolor (Vuill.) Tiraboschi	63-59
A.flavus Link v.columnaris Raper and Fennell	66-6
A.ustus (Bain) Thom and Church	64-37
A.terreus Thom	65-32
A.wentii Wehmer	66-1
A.ochraceus Wilhelm	65-92
A.niger Van Tieghem	63-68
Penicillium sp.	65-60
Penicillium sp.	63-123
Penicillium sp.	63-207
Penicillium sp.	63-105

Stock slant cultures of the above mentioned fungi were maintained on Czapek Dox agar medium. They were subcultured approximately every three months by loop transfer and incubated at 30°C for 5-10 days, until sporulation occurred. They were then stored in a refrigerator at 5°C.

2) Spore suspensions

The fungi were incubated in 125 ml Erlenmeyer flasks, containing 20 ml of Czapek Dox agar medium, for two weeks at 30°C. A 10 ml aliquot of sterilized, demineralized water with 2 drops/100 ml of Tween 80 (Difco Laboratories) as a wetting agent, was pipetted into each Erlenmeyer flask, containing a sporulating culture. A spore suspension was obtained by gently swirling the contents on a magnetic stirrer. A 10 ml aliquot of water was added at this stage to facilitate subsequent repeated filtrations. The suspension was filtered through several layers of sterilized cheese-cloth to remove conidiophores, mycelial fragments and spore clumps. The filtered suspension was centrifuged at 5000 rpm and the spores were washed with sterilized, demineralized water, recentrifuged and washed again to remove adhering nutrients. The pellet of spores, obtained after the third centrifugation, was dispersed and diluted to the desired concentration in sterilized, demineralized water containing Tween 80. Spore concentration was determined with a hemocytometer counting chamber.

The suspension was examined with a microscope and considered satisfactory for irradiation experiments only when spore clumps and mycelial fragments were absent. If spore clumps were present at this stage, they were dispersed by adding glass beads and swirling the suspension on a magnetic stirrer.

3) Irradiation and plating

All irradiation treatments were carried out in a Gammacell 220 (91) at the Radiation Laboratory of the University of Alberta. With each set of experiments the dosimetry was done using a Frickle Ferrous Sulfate dosimeter (3, 5, 128).

A spore concentration of 10^4 - 10^6/ml was used for irradiating the species of Aspergillus and Penicillium. Six sterilized, 125 ml Erlenmeyer flasks, each containing 20 ml of the spore suspension,were used for irradiation. The contents of each of 5 flasks were irradiated with doses of 25, 50, 75, 100, and 125 krad at a dose rate of 7.2 krad/ minute. The sixth flask served as a non-irradiated control. Immediately after irradiation the flasks were placed in an ice bath. The irradiated suspensions were then diluted to contain approximately 1500 spores/ml. An aliquot of 0.1 ml of this diluted suspension was pipetted into each of 10 Petri plates. Five ml of Czapek Dox agar medium, liquified at 45°C, was added to each plate and shaken gently to obtain an even dispersion of spores. These cultures were incubated for 3 days at 30°C. The ability of a spore to form a visible colony was taken as the criterion for survival. The colonies were counted under a colony counter (10 X magnification).

B. Radiosensitization of Aspergillus flavus

1) Organism

A. flavus Link v.columnaris Raper and Fennell, culture

no. 66-6, was used throughout these investigations (see previous section).

2) Spore suspensions

Stock cultures were maintained, and spore suspensions were prepared in a manner described previously. Seven-day old sporulating cultures were used in all experiments. To avoid any swelling of spores, the suspensions were processed as soon as possible after harvest.

3) Preparation of mycelium

One ml aliquots, containing 2×10^3 - 4×10^3 spores/ml from the stock spore suspension of A.flavus, were added to 80 ml of Czapek liquid medium in 250 ml Erlenmeyer flasks. The spores in this liquid medium were then incubated on a rotary culture shaker at room temperature ($25^{\circ}C$) for 24 hours at an incandescent light intensity of 90 foot-candles. The contents of 5 flasks were combined and the liquid medium was decanted. The mycelial suspension was washed approximately 10 times with sterilized, demineralized water, containing 2 drops/100 ml of Tween 80, to remove the adhering nutrients. The germinating spores were centrifuged at low speed and resuspended in sterilized, demineralized water. The structures,so obtained,appeared as aggregates of germinating spores, but they could be easily separated into individual germinating units by shaking in water. The concentration of these mycelial units was determined by culturing 1 ml of the suspension on Czapek Dox agar medium.

4) Irradiation of spores in presence of chemicals

a) Chemicals

The following chemicals, in the concentrations indicated were tested as radiosensitizers of spores of A. flavus:

Chemical	Concentration	Source
1. Sodium chloride, NaCl	5 %	Fisher Scientific Co.
2. Sodium bromide, NaBr	5 %	"
3. Sodium iodide, NaI	5 %	"
4. Iodized salt	5 %	Sifto Salt Co. Montreal.
5. Potassium chloride, KCl	5 %	Fisher Scientific Co.
6. Potassium bromide, KBr	5 %	"
7. Potassium iodide, KI	5 %	"
8. Calcium chloride, $CaCl_2$	5 %	"
9. Calcium bromide, $CaBr_2$	5 %	Allied Chemical and Dye Corp. N.Y.
10. Calcium iodide, CaI_2	5 %	K and K Labs. N.Y.
11. Calcium nitrate, $Ca(NO_3)_2$	5 %	Fisher Scientific Co.
12. Iodoacetamide, ICH_2COONH_2	0.001 %	J.T.Baker Chemical Co.
13. Iodoacetic acid, ICH_2COOH	0.1 %	"
14. Vitamin K_5, $NH_2C_{10}H_5(CH_3)OH$ HCl	0.01 %	Eastman Organic Chemicals
15. Potassium nitrite, KNO_2	5 %	Fisher Scientific Co.
16. Sodium nitrite, $NaNO_2$	5 %	"
17. Sodium nitrate, $NaNO_3$	5 %	"

b) Irradiation and plating

The chemicals were weighed in aluminum dishes and dissolved in sterilized, demineralized water to give the desired concentration. One ml of the suspension, containing 10^5 spores, was added to a 10 ml solution of the chemical in a 125 ml sterilized Erlenmeyer flask. The spores were kept in the chemical for a period of 17.3 minutes, which was the period of time required for the administration of the maximum dose (125 krad). Irradiation was applied in the last portion of the period of chemical treatment, so that both treatments terminated simultaneously. A set of 7 flasks was prepared for each chemical. Two of these flasks served as control, one with spores suspended in distilled water and non-irradiated, and one with spores in a chemical solution kept for a period of time equal to that required for the administration of maximum radiation dose, but remaining non-irradiated. Each of the 5 flasks, in which the contents were irradiated, received a dosage of 25, 50, 75, 100 or 125 krad at a dose rate of 7.2 krad/minute.

Immediately after irradiation the suspension was diluted to a concentration of 1×10^3 spore/ml. Aliquots of 0.1 ml per plate were poured to give approximately 10^2 colonies. Ten plates were poured for each treatment. During any period of delay between irradiation and plating, the samples were kept in an ice bath. Subsequent plating and counting procedures were the same as previously described.

5) Irradiation of mycelium in presence of chemicals

a) Chemicals

The chemicals and concentrations were the same as those used in experiments with spores of A.flavus.

b) Irradiation and plating

Mycelial units could not be kept for more than 64 hours at 4°C, because they tended to coalesce into inseparable clumps. Fresh mycelial suspensions were, therefore, used for each experiment. The experimental procedure was similar to that used for spores,with minor modifications. The mycelial units were held in suspension by swirling on a magnetic stirrer. An aliquot of 2 ml of this suspension, containing 10^3 mycelial units, was pipetted into 10 ml of chemical solution in a 125 ml Erlenmeyer flask. Seven flasks were used for each chemical and the treatments were similar to those used for spores. After irradiation the mycelial units were centrifuged at 2000 rpm and the units were repeatedly washed with sterilized, demineralized water. This procedure was repeated for each treatment.

The mycelial suspension,obtained after washing,was resuspended in 10 ml of sterilized, demineralized water and dispersed in aliquots of 1 ml per plate. Five ml of Czapek Dox agar medium was added at 45°C and the plates were shaken gently to uniformly distribute the mycelial units throughout the medium. After incubating the cultures at 29°C for 2 days, the number of colonies was recorded in in each treatment.

C. Heat-Irradiation Interactions in Spores and Mycelium of A.flavus

1) Spores

a) Heating

Spore suspensions,obtained by a method previously described, were used. Heat treatments were done in a constant temperature water bath (Thelco, Model 83, Precision Scientific Co., U.S.A.). The spores were heated at 25°C (room temperature), 35°, 40°, 45°, 50°and 55°C. Aliquots of 5 ml of sterilized, demineralized water, in 25 ml Erlenmeyer flasks, were brought to the desired temperature in the water bath. One tenth of a ml of spore suspension, containing 10^3 spores/ml, was added to each flask and held at the desired temperature for 20 minutes. The temperature of the solution inside the flask was recorded with a thermocouple rod of a thermograph. After heating, the temperature of the solution was lowered quickly by immersing the flask in an ice bath and by adding 5 ml of ice-cold, sterilized, demineralized water to the flask contents. The spores that had undergone the heat treatment were either plated for survival studies or further treated as described in future sections.

b) Heating followed by irradiation

After the heat treatment and cooling, the spores were immediately irradiated with doses of 25, 50, 75, 100 and 125 krad in a Gammacell 220, at a dose rate of 6.25 krad/minute and plated by a method previously described. There

were two controls for every experiment, one containing only non-irradiated and non-heated spores and the second containing non-irradiated, heated spores.

In order to study the effect of the time interval between heating and irradiation on the radiosensitivity of the spores, the experiments were conducted as described above, with the difference that heating and irradiation were separated by a time interval of 24 and 48 hours. During this interval of time the spore suspensions were held at 25°C.

c) Irradiation followed by heating

The methods were similar to those described for pre-heat and post-irradiation studies, except that the treatments were reversed so that heating followed irradiation. Time intervals of 24 and 48 hours were also used between irradiation and heating in another set of experiments.

d) Simultaneous heating and irradiation treatment

An arrangement for heating the spores inside the Gammacell irradiation chamber was improvised (Fig. 1). Water was heated in a thermostatically-controlled bath and was circulated through the irradiation chamber by a small pump, fitted inside the water bath. A glass tube coil, placed in a 100 ml beaker, surrounded by water, served as a heat exchanger. Forty ml of sterilized, demineralized water in the beaker was maintained at the desired temperature inside the irradiation chamber by means of the heat exchanger. A 5 ml aliquot of spore suspension was

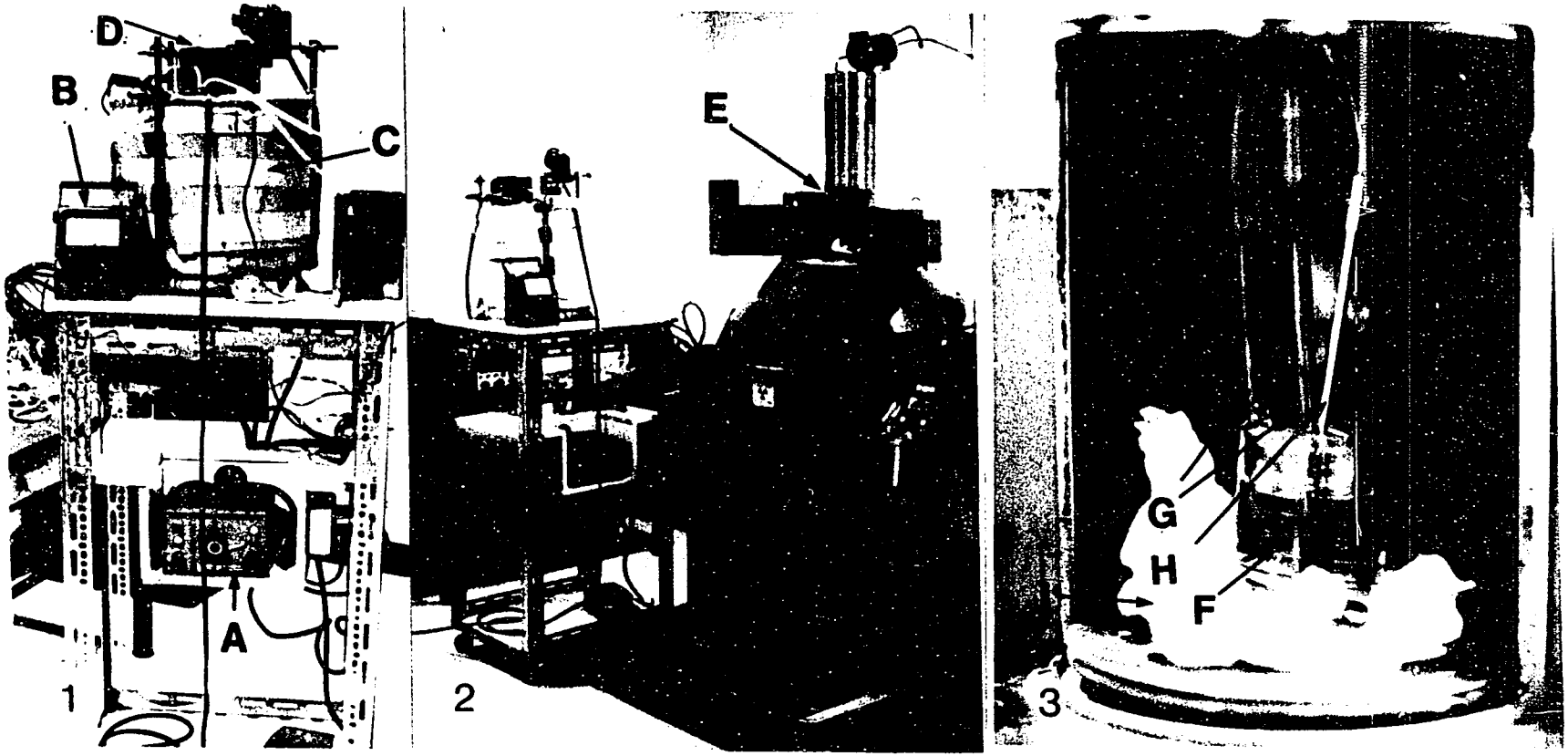

FIG. 1. Arrangement for heating spores or mycelium of A.flavus inside the irradiation chamber of a Gammacell 220. 1: A close view of the heating unit. A)Thermoregulator. B)Thermograph for recording the inside temperature. C)Water bath. D)Motor for pumping water inside the irradiation chamber. 2: Heating unit attached to the Gammacell. E)Irradiation chamber. 3: Detailed view of the irradiation chamber. F)Heat exchanger. G)Terminals of the heat exchanger. H)Thermocouple rod. I)Piece of cotton for insulating the bottom of the beaker.

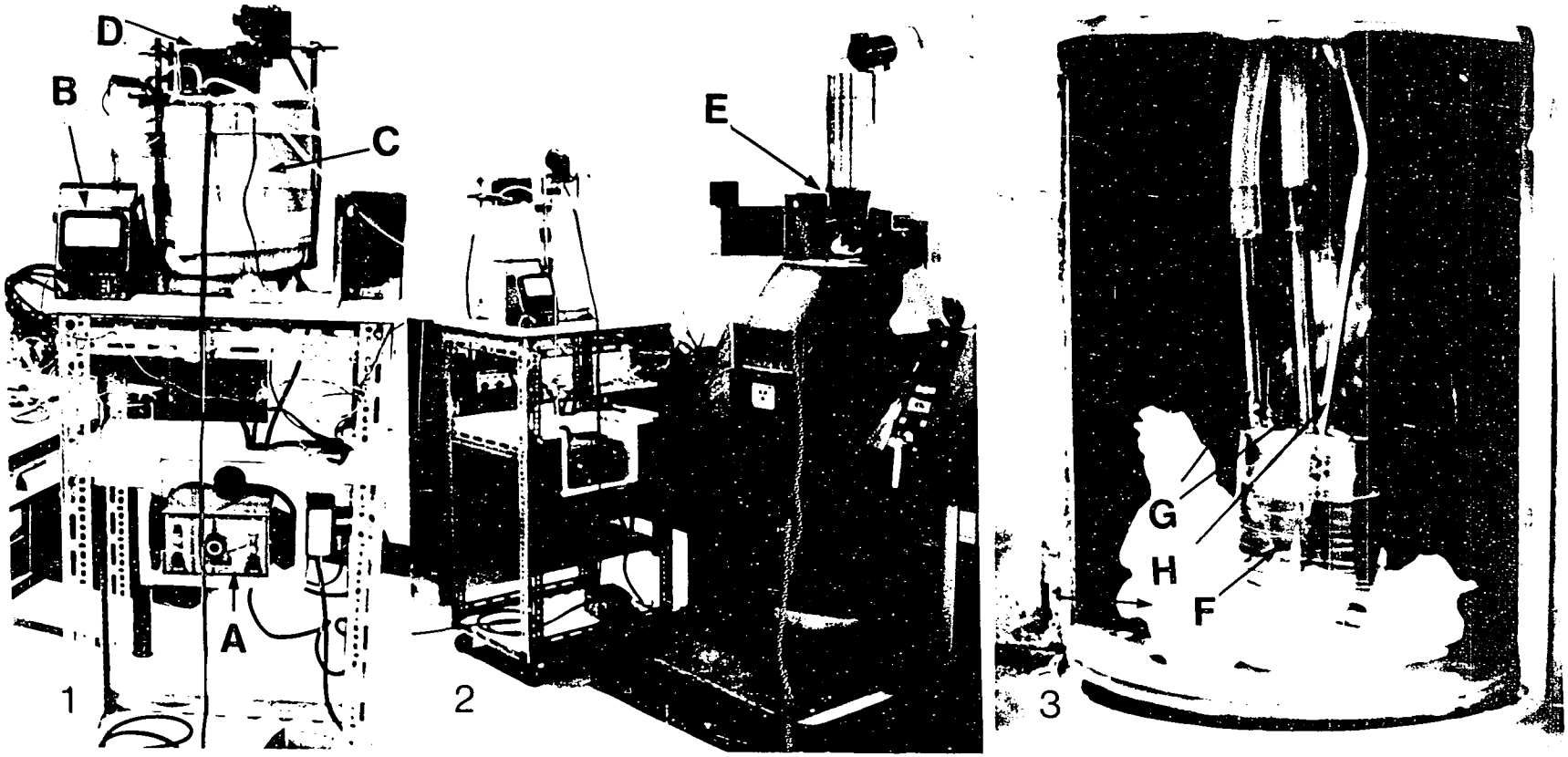

FIG. 1. Arrangement for heating spores or mycelium of A.flavus inside the irradiation chamber of a Gammacell 220. 1: A close view of the heating unit. A)Thermoregulator. B)Thermograph for recording the inside temperature. C)Water bath. D)Motor for

added, and heating at the desired temperature was maintained for 20 minutes. The material was irradiated to provide a dosage of 25, 50, 75, 100 and 125 krad. Since a period of 20 minutes was required to deliver a dose of 125 krad, therefore, where a dosage of less than 125 krad was needed the irradiation was started at the appropriate time following the initiation of heating, so that both treatments were terminated at the same time.

After these treatments, 40 ml of ice-cold sterilized, demineralized water was added and the container placed in an ice bath. Subsequent dilution and plating procedures were similar to those previously described.

2) Mycelium

a) Heating

Heat treatments were similar to those for spores, except that 1 ml aliquots of suspension, containing 10^3 mycelial units, were used.

b) Heating followed by irradiation

Similar treatments, same temperatures and same irradiation doses were used as described for spores. In experiments involving a 48 hour interval between heating and irradiation, the mycelial clumps were separated by adding glass beads and swirling on a magnetic stirrer.

c) Irradiation followed by heating

These treatments were the same as described for spores, with the difference that, in the samples which were heated 48 hours after irradiation, mycelial clumps were

separated by the use of glass beads, as mentioned above

d) Simultaneous heating and irradiation treatment

The same arrangements and the same procedure was used as described for the spores, except that, at the end of treatments, the mycelium was centrifuged at low speed and resuspended in 10 ml of sterilized, demineralized water.

D. Survival Curves

The ability of spores or mycelium to form visible colonies was taken as the criterion for survival following irradiation or heat damage. The percentage of survival was calculated with the help of an APL Computer (44) using the equation:

$$\frac{\text{Average no. of colonies on 10 plates from treated suspensions (Irradiation or heat).}}{\text{Average no. of colonies on 10 plates from non-treated (control) suspension.}} \times 100$$

The survival curves were also prepared, using an APL Computer, by plotting the percentages of survival as a function of γ-irradiation dose (in krad) on a semilog-arithmic paper.

E. Statistical Analyses

"t" values of significance at 1 % level for each treatment were calculated with the help of an APL Computer.

RESULTS

The "t" values for differences between treatments have been calculated at the 1 % level. Therefore all differences, reported in the results that follow, are of the highly significant order.

All the survival curves are sigmoidal in shape with a shoulder and an exponential region, unless otherwise stated.

A. Survival Curves of *Penicillium* and *Aspergillus* species

The 4 species of *Penicillium* differed significantly in resistance to gamma irradiation, when their LD_{37} values were compared (Fig. 2). *P.* sp., 65-60, was the most resistant with an LD_{37} of 71 krad, while *P.* sp., 63-207, was the most sensitive with an LD_{37} of 33 krad. A sharp decline in the exponential portion of the survival curve of *P.* sp., 63-105, was noticeable,so that at 125 krad this species was the most sensitive.

The species of *Aspergillus* showed different degrees of resistance to gamma irradiation when their LD_{37} values were compared (Fig. 3). The most resistant species was *A. terreus* with an LD_{37} of 54 krad, while the most sensitive species was *A.ochraceus* with an LD_{37} of 31 krad. The LD_{37} values of other species were not significantly different from each other. At a dose of 125 krad, however, a different pattern of resistance emerged, primarily due to a sharp decline in the exponential portion of the survival curves of *A.flavus*, *A.wentii* and *A.versicolor*. Thus, at 125 krad, *A.flavus* and *A.versicolor* had similar survival

values, but A.niger was significantly more resistant than A.flavus. Similarly, A.ustus showed more resistance than A.flavus.

B. Radiosensitization of Spores of A.flavus

Most chemical compounds, used for radiosensitization, were not toxic to the spores of A.flavus in the concentration used, but $NaNO_3$ and vitamin K_5 resulted in 80 % and 68% survival, respectively, at zero dose (Figs. 4-9).

The presence of NaCl, NaBr or NaI solutions during irradiation of spores resulted in a significant reduction in survival, when compared to the control (Fig. 4). The degree of sensitization, effected by these compounds, was in the order NaI > NaBr > NaCl. An increase in the sensitization capacity of these chemicals occurred with each increase in the radiation dose. At 25 krad, NaCl did not contribute to a significant reduction in survival, while NaBr and NaI did. At the same dose the difference between the effects of NaI and NaCl was significant, while no difference was observed with NaBr treatment. However, at 50 krad and above, survival at each dose was considerably different than survival at any other dose.

Iodized salt proved to be effective as a radiosensitizer (Fig. 5). The difference in the effect on spore survival by NaCl and iodized salt was significant only at 100 krad, at which dose 0.11 % and 0.01 % survivals were obtained, respectively.

Potassium chloride, KBr and KI were good radio-

sensitizers of spores of A.flavus at all the irradiation doses applied (Fig. 6). The survival curves for KBr and KI were superimposable, and both of these compounds showed a significant sensitization at 75 krad, when compared to KCl. A reduction in the shoulder region was seen for the survival curves of spores irradiated in KBr and KI solutions, indicating less resistance at the lower doses.

Compounds containing calcium resulted in a protective rather than a sensitizing effect on spores treated with an irradiation dose of 25 krad, but their effects varied at higher doses (Fig. 7). At the higher doses, a sharp decline in the shoulder regions of the survival curves for $CaBr_2$ and CaI_2 indicated a significant sensitization of the spores. Calcium chloride and $Ca(NO_3)_2$ resulted in a significant protective effect, with $Ca(NO_3)_2$ conferring more protection than $CaCl_2$. Calcium iodide had a higher sensitizing effect than $CaBr_2$ at 50 and 75 krad. At 100 krad, a complete kill of spores occurred in both chemical solutions.

Sodium nitrite and $NaNO_3$ were radiosensitizers of spores of A.flavus, but KNO_2 did not affect the shape of the survival curve (Fig. 8). Sodium nitrite had a greater sensitizing capacity than $NaNO_3$ at 75 and 100 krad.

Iodoacetic acid, iodoacetamide and vitamin K_5 were strong radiosensitizers, with iodoacetic acid being the most effective, followed by the others in the above order (Fig. 9). The survival curve for iodoacetic acid was almost exponential in shape, while in iodoacetamide a small

shoulder region occurred, indicating some resistance at the lower doses.

C. Radiosensitization of Mycelium of A.flavus

Spores of A.flavus, which were incubated at room temperature in liquid Czapek medium for 6 hours before irradiation, were more radiosensitive than those which were irradiated without prior incubation, but this sensitivity was reversed for spores incubated for 12 or 24 hours (Fig. 10.). After 6 hours of incubation the spores swelled, and germ tubes became barely obvious in 12 hours. In 24 hours of incubation, a 2-celled germ tube was produced. These germinated spores tended to clump into visible mycelial balls. However, at this stage of growth, the clumps could be easily separated into individual germinating spore units. Any extension of incubation beyond 24 hours resulted in an intertwining of hyphae to produce clumps of mycelium which could not be separated into their components. Resistance to irradiation continued to increase with increase in incubation time. After 60 hours the mycelium was extremely resistant to irradiation with an LD_{37} of 181 krad (Fig. 11).

Sodium chloride and NaI were highly effective as radiosensitizers of mycelium at each dose level, while NaBr had the reverse effect by acting as a protectant (Fig. 12). These compounds showed toxicity, in the order of NaI > NaBr > NaCl. Iodized salt was also toxic to the mycelium and had a sensitizing effect only at a dose of 125 krad (Fig. 13). This effect was significantly less when compared to that of

NaCl at the corresponding dose.

Potassium chloride, KBr and KI were highly effective as radiosensitizers, and also exhibited toxicity at the concentration used (Fig. 14). The order of radiosensitizing capacity was KI > KBr > KCl. A difference between the sensitizing capacity of KCl and KBr occurred only at 125 krad, while KI differed significantly from the other salts at 75 krad and above. In the KI solution, a dose of 100 krad was required to achieve a complete kill of the mycelium, while in KBr the same effect required a dose of 125 krad.

Among the calcium compounds, CaI_2 was the most effective radiosensitizer and the behaviour of other calcium compounds varied with each dose (Fig. 15). The radiosensitizing effect of $CaCl_2$ was maximum at 125 krad. Calcium nitrate behaved as a protective agent at doses up to 75 krad, but at higher doses there was a sensitizing effect. In calcium bromide, the resistance of the mycelium was increased, as is evidenced by the broadening of the shoulder region in the survival curve. These compounds also exhibited toxicity.

Sodium nitrate, $NaNO_2$ and KNO_2 radiosensitized the mycelium of A.flavus (Fig. 16). The radiosensitizing capacity of these compounds was most pronounced at 125 krad, with the order of the effect being $KNO_2 > NaNO_3 > NaNO_2$. However, at 75 krad, only $NaNO_3$ was able to sensitize the mycelium. These compounds also showed toxic effects at

the concentration used, with the degree of toxicity being in the order of $NaNO_3 > KNO_2 > NaNO_2$.

Iodoacetic acid, iodoacetamide and vitamin K_5 radiosensitized the mycelium of A.flavus, with the first one being the most effective (Fig. 17). The presence of iodoacetic acid during irradiation resulted in an exponential survival curve with no shoulder. Each of these compounds showed a different degree of toxicity, with the order being iodoacetic acid > iodoacetamide > vitamin K_5.

D. Combined Effect of Heat and Irradiation on Survival of Spores and Mycelium of A.flavus

Heating spores or mycelium of A.flavus at 35°C for 20 minutes did not adversely affect their survival, but exposure for the same period to a temperature of 55°C resulted in only 45 % and 35 % survival for spores and mycelium, respectively (Fig. 18). Mycelium was significantly more heat-resistant than spores at 45°and 50°C, while the opposite was true at 55°C. Mycelium was also more resistant than spores to gamma irradiation at each dose level.

Irradiation of spores at temperatures between 35°C and 55°C was considerably more effective as a fungicidal treatment than irradiation or heat alone, with this effect being more pronounced at higher temperatures (Fig. 19). The degree of synergism conferred by the different temperatures was in the order of 55°> 50°>45°>40° = 35° > 25°C. The survival curve, obtained at 35°C, showed a significant difference from that of control at and above an

irradiation dose of 75 krad. Irradiation at 40°C had a synergistic effect at all dose levels, when compared to survival after irradiation at room temperature (25°C), but there was no difference between the curves for 35°C and 40°C. Gamma irradiation survival values at 45°C were significantly less when compared to those at 40°C, except at 125 krad. A reduction in the shoulder area and a steep decline in exponential region were characteristic for irradiation treatment at 50°C, while an exponential survival curve developed at 55°C.

An increase in temperature, at which mycelium was irradiated, increased the lethal effects of irradiation (Fig. 20). The synergistic effects of various temperatures were in the order of 55°>50°>45°>40°>35°= 25°C. Irradiation at 35°C did not change survival when compared to that at 25°C, but at 40°C, irradiation lowered survival significantly from that occurring at 35°C. Similarly, with each subsequent 5°C rise in temperature, there was a marked decrease in survival. At 50°C, the gamma irradiation survival curve had a sharply declining exponential portion with a less pronounced shoulder, while at 55°C, the curve approached an exponential shape.

Heat treatments were combined with irradiation in three different ways, i.e., immediately before irradiation, during irradiation or immediately after irradiation. Different sequences of heat at 35°C and irradiation treatment had different effects on survival at certain doses (Fig. 21).

Heating the spores at 35°C for 20 minutes did not sensitize them to inactivation by doses below 125 krad. When irradiation was applied first, it did not predispose the spores to heat inactivation. A simultaneous application of heat at 35°C and irradiation at 75-125 krad had a synergistic effect when compared to each of the other two combinations.

A 40°C heat treatment predisposed the spores to irradiation inactivation, as shown by a pronounced change in the slope of the survival curve (Fig. 22). Thus, at doses of 50 krad and above, the survival of heated spores was considerably lower than would have been expected if the two treatments were only additive in their effect. Irradiation at 50 krad and above also sensitized the spores to heating at 40°C, but this sensitization was less when heating preceded irradiation. A simultaneous application of irradiation and heat at 40°C was as effective in inactivating the spores as irradiation followed by heat, except that, at 125 krad, the simultaneous treatment was more effective in reducing spore survival.

Treatments, involving the heating of spores at 45°C, either before, after or during irradiation, were highly synergistic in reducing spore survival when compared to the effect of irradiation or heat alone (Fig. 23). The degree of synergism in these treatments between 25-75 krad was in the order 45°C + irradiation > irradiation + 45°C > irradiation at 45°C. Similarly, various pre-, post-or during-irradiation combinations with a temperature of 50°C showed

a highly synergistic effect when compared to irradiation or heat alone (Fig. 24). This effect was evident at all dose levels. A sequence, involving heating at 50°C followed immediately by irradiation, was the most effective in the synergistic inactivation of the spores. Simultaneous heat and irradiation treatments were similar in their effect to that of irradiation followed by heating at 50°C, except at 125 krad, where the former was more effective.

Heating at 55°C either before, after or during irradiation was highly synergistic treatment for the inactivation of spores of A.flavus (Fig. 25). Pronounced changes occurred in the shapes of the survival curves. Among the various combinations, heating the spores before irradiation and a simultaneous application of these two treatments, were the most effective. A low dose of 25 krad resulted in 1.5 % survival,while at 50 krad there were no survivors. The survival curves for these two treatments were superimposable and were nearly exponential. Irradiation strongly sensitized the spores to inactivation by heat when compared to irradiation alone, but this sensitizing effect was significantly less when compared to the other two treatments. The survival curve of this treatment had a reduced shoulder and a sharply declining exponential portion.

Heating mycelium at 35°C predisposed it to inactivation by irradiation at 75-125 krad, but this effect was not evident at lower doses (Fig. 26). By contrast, irradiation did not sensitize the mycelium to heat inactivation, and

the same was true for a simultaneous application of irradiation and heat.

A heat treatment of 40°C, either immediately followed by irradiation, or during irradiation, or after irradiation at 100 and 125 krad, showed a synergistic effect when compared to irradiation alone (Fig. 27). The synergistic effect of heating before irradiaton or after irradiation was similar. Irradiation of mycelium at 40°C at 125 krad had a synergestic lethal effect.

A simultaneous application of heat at 45°C and irradiation showed a remarkable synergism at each of the dose levels (Fig. 28). Thus, irradiating mycelium at 45°C resulted in an inactivation which was far greater than would have been expected if the two treatments were only additive in effect. Similarly, heating the spores at 45°C predisposed them to inactivation by irradiation when compared to irradiation alone. However, at 100 and 125 krad, the simultaneous application of heat and irradiation was more effective. Irradiation did not sensitize the mycelium to inactivation by heat at 45°C.

Various pre-,post-or during-irradiation combinations with a temperature of 50°C had a highly synergistic lethal effect when compared to either treatment applied separately (Fig. 29). This effect was evident at all dose levels. Simultaneous application of heat and irradiation was the most effective in the synergistic inactivation of the mycelium. Heating at 50°C predisposed the mycelium to irradiation

inactivation. Similarly, irradiation also sensitized the the mycelium to heat inactivation, but this sensitization was much less when compared to the other two combined treatments.

Heating at 55°C, either before, during or after irradiation, was extremely effective synergistic treatment as evidenced by pronounced changes in the shape of the survival curves (Fig. 30). A simultaneous application of heat and irradiation was the most effective sensitizing combination. In this treatment, the shape of the survival curve was almost exponential with no shoulder region. The less effective combination was heating the mycelium followed by irradiation, and the least effective was irradiation before heating. The survival curves for these treatments had a much reduced shoulder and a sharply declining exponential portion.

E. Effect of Time Interval between Heat and Irradiation on the Sensitization of Spores and Mycelium of A. flavus

Spores or mycelia of A.flavus which were seeded on Czapek agar medium 24 or 48 hours after irradiation were capable of repairing the irradiation damage resulting in an increase in the number of survivors,when compared to those which were seeded immediately after irradiation (Figs. 31, 32). The survival values at 25 or 50 krad after 48 hours were the same as after 24 hours, but at higher doses the survival was significantly greater after a 48-hour interval between irradiation and plating.

A time interval between heating and irradiation of the spores resulted in a desensitizing effect when compared to irradiating immediately after heating. Heating the spores at 35°C, immediately followed by irradiation, had a synergistic effect, but, when the time interval between heating and irradiation was 24 hours, survival increased significantly at all dose levels (Fig. 33). An interval of 48 hours resulted in more survivors at 75-125 krad only.

Heating the spores at 40°C sensitized them to irradiation which was applied immediately after the heat treatment, but an interval of 24 or 48 hours between the two treatments increased survival significantly (Fig. 34). Heat treatment of 45°C in association with irradiation resulted in survival curves similar to those obtained with treatment at 40°C (Fig. 35).

Heating the spores at 50°C or 55°C, followed immediately by irradiation, showed a highly synergistic lethal effect, but a time interval of 24 or 48 hours between these treatments resulted in a significant decrease in the sensitization of spores (Figs. 36 , 37). Survival after 48 hours was greater than after 24 hours. At these temperatures, however, at doses exceeding 100 krad, no increase in survival was observed.

Treatments involving irradiation of spores, followed by heating at various temperatures, also showed similar desensitizing effects as a result of increasing the time interval between the two treatments.

Irradiation of spores, followed immediately by a heat treatment of 35°C, did not have a synergistic effect, but,when the spores were heated 24 or 48 hours after irradiation, survival was significantly increased (Fig. 38). Similarly, a time interval of 24 or 48 hours between irradiation of spores and a heat treatment at 40°C showed an increase in survival (Fig. 39).

Heating the irradiated spores at 45°C was a highly synergistic lethal treatment, but heating the spores 24 hours after irradiation was not as effective a sensitizing treatment as the first one (Fig. 40). A time interval of 48 hours increased the survival of spores and this increase was significantly different from that after 24 hours,only at 125 krad.

An interval of 24 hours between irradiation of spores and application of a 50°C temperature showed an increase in survival only at lower doses of 25 or 50 krad (Fig. 41). However, after 48 hours some repair occurred at higher doses as well. Similar results were obtained when irradiation was followed by heating at 55°C (Fig. 42).

Heating mycelium, immediately followed by irradiation, had a synergistic lethal effect, but delaying the application of irradiation by 24 or 48 hours resulted in a desensitization.

Mycelium, heated at 35°C,followed by irradiation after a delay of 24 hours, showed a significant increase in survival, when compared to survival that occurred when irradiation followed heat immediately (Fig. 43). Extension

of this time interval to 48 hours resulted in increased survival at doses above 50 krad. However, repair of damage was complete at lower doses after 24 hours.

Mycelium, heated at 40°C, followed by irradiation after 24 hours, did not show any difference in survival in relation to survival when irradiation was applied immediately (Fig. 44). However, a time interval of 48 hours between heating and irradiation resulted in a significant increase in survival.

Heating the mycelium at 45°C, followed immediately by irradiation,was asensitizing treatment, but application of irradiation after 24 or 48 hours resulted in a desensitizing effect, with this effect being greater after 48 hours than after 24 hours (Fig. 45).

A time interval of 24 hours between the application of a 50°C heat treatment and irradiation resulted in a considerable increase in survival, especially at 25 and 50 krad (Fig. 46). After 48 hours the survival was not significantly different than survival after 24 hours. A similar type of response was shown by a heat treatment of 55°C followed by irradiation after 24 hours (Fig. 47).

Similarly, a time interval of 24 or 48 hours between irradiation of mycelium and heating at various temperatures resulted in an increase in the number of survivors. However, at higher irradiation doses and at higher temperatures this effect did not occur.

Irradiation of mycelium, followed by a temperature of

35°C after 24 or 48 hours resulted in an increase in the number of survivors, with no difference between the 2 intervals (Fig. 48).

Irradiation, followed by a heat treatment of 40°C after 24 hours, resulted in a considerable increase in survival at doses up to 75 krad, while at 125 krad no such increase was seen (Fig. 49). Survival increased further when there was a time interval of 48 hours between the two treatments.

Irradiation of mycelium, followed by heating at 45°C after 24 or 48 hours, resulted in an increase in survival (Fig. 50). Survival after 48 hours was significantly greater than survival after a 24-hour interval.

A temperature of 50°C, applied 24 hours after irradiation, resulted in greater survival than that resulting from heat being applied immediately after irradiation (Fig. 51). Similarly, a 50°C treatment, 48 hours after irradiation, resulted in more survivors than after 24 hours.

A heat treatment of 55°C, applied immediately after irradiation of the mycelium, showed a synergistic lethal effect, but a delay of 24 or 48 hours resulted in an increase in the number of survivors at 25 or 50 krad only (Fig. 52).

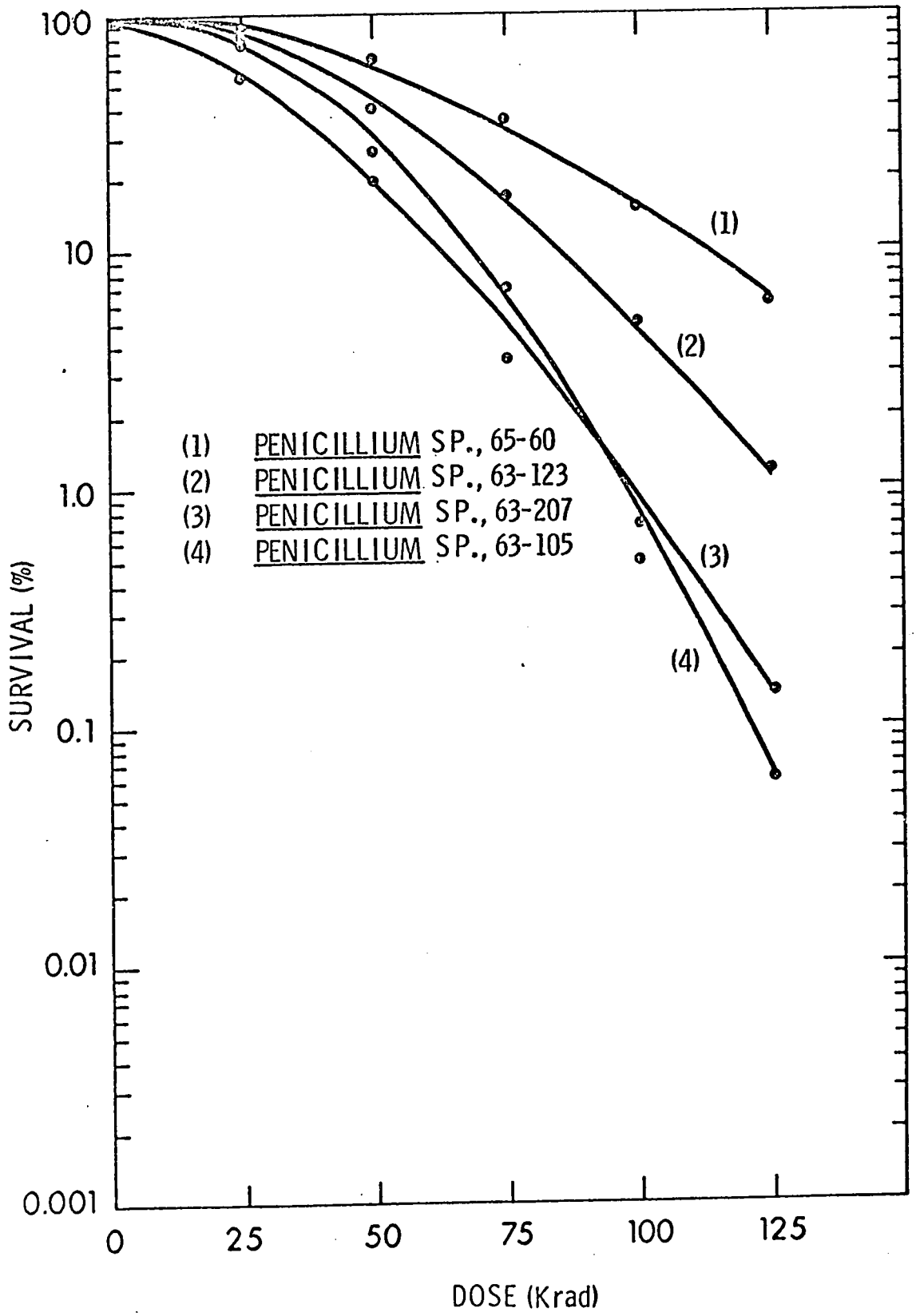

FIG. 2. γ-irradiation dose response curves for species of Penicillium.

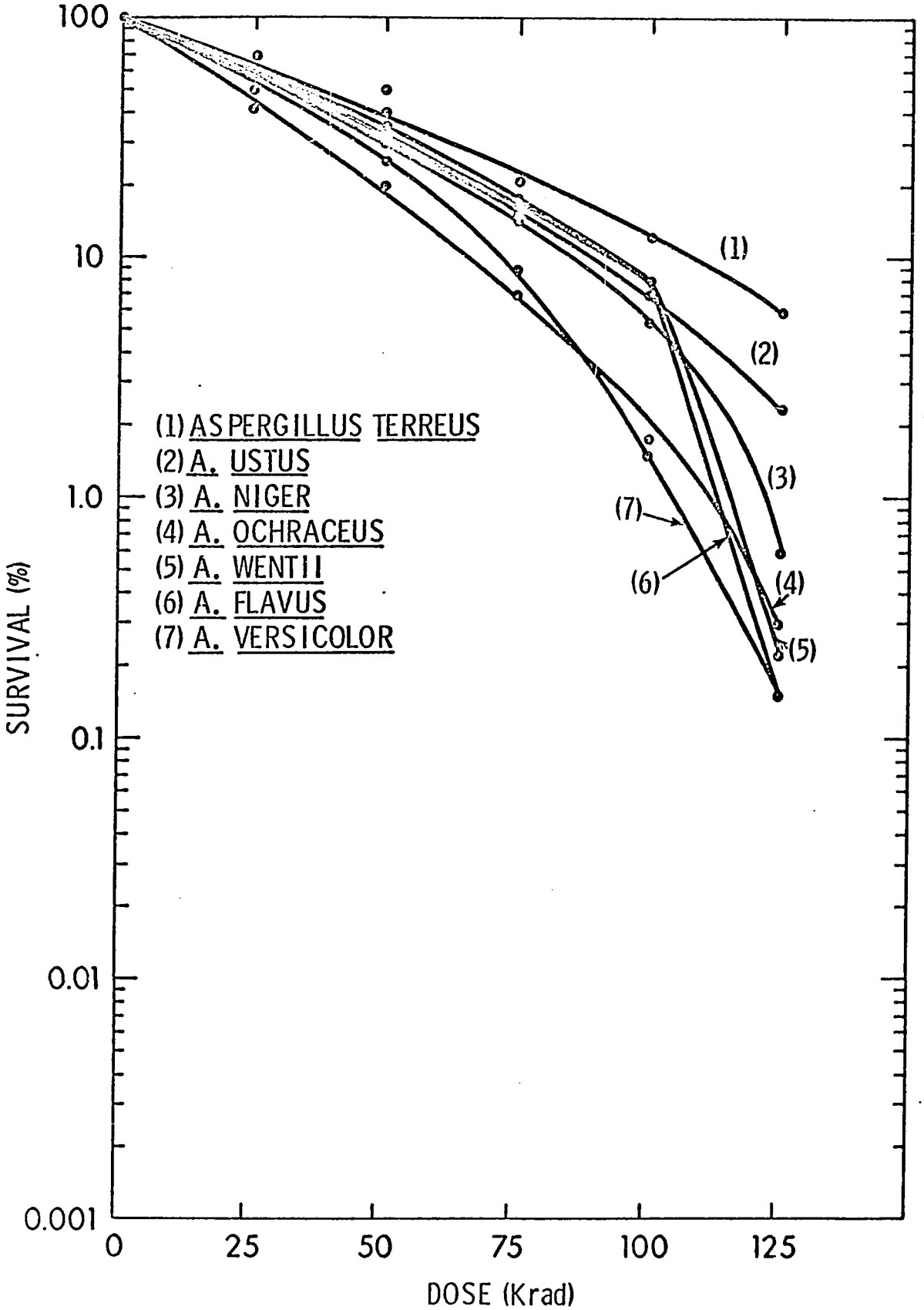

FIG. 3. γ-irradiation dose response curves for species of Aspergillus.

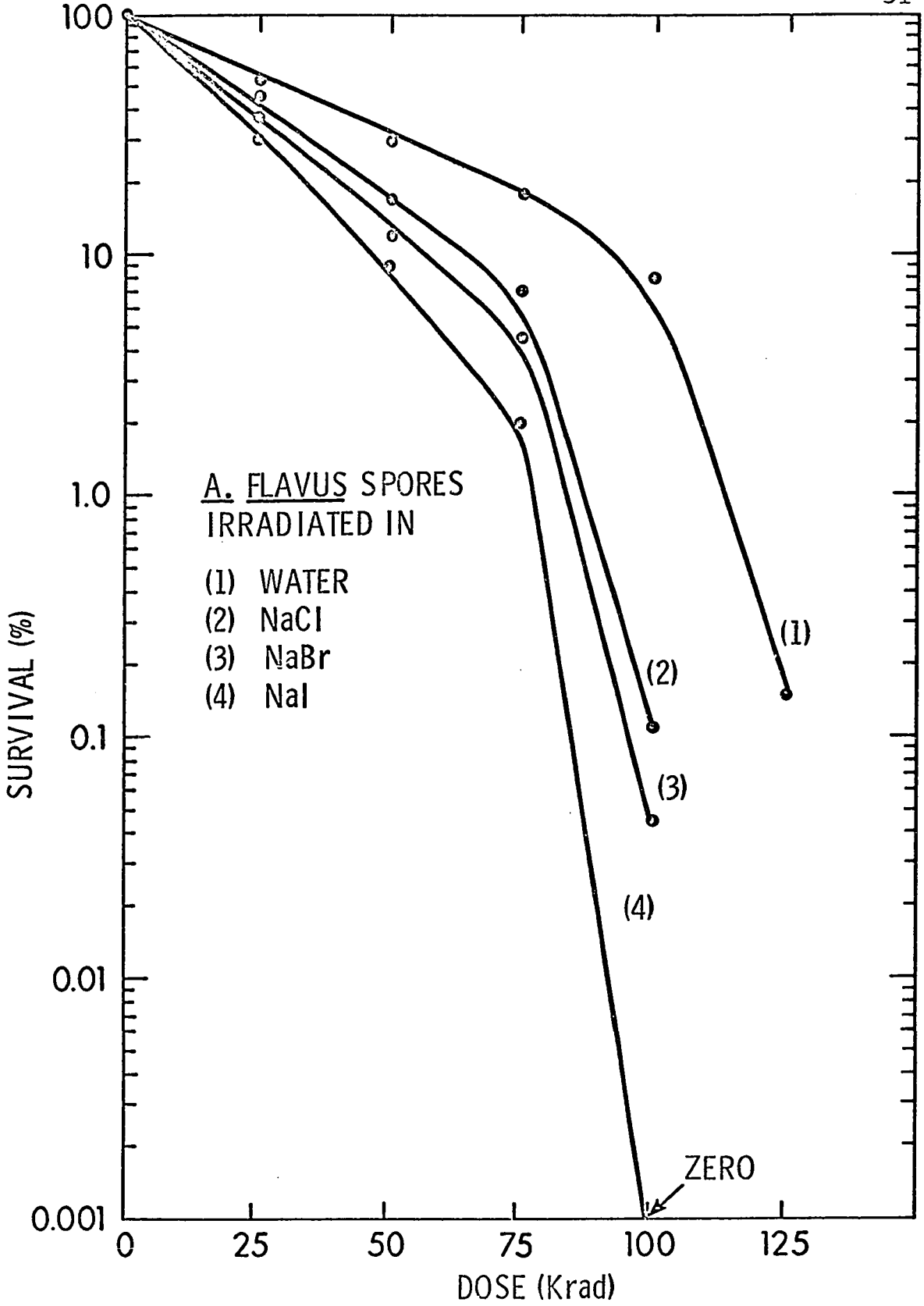

FIG. 4. ϒ-irradiation survival curves for spores of A.flavus, irradiated in a solution of NaCl, NaBr or NaI.

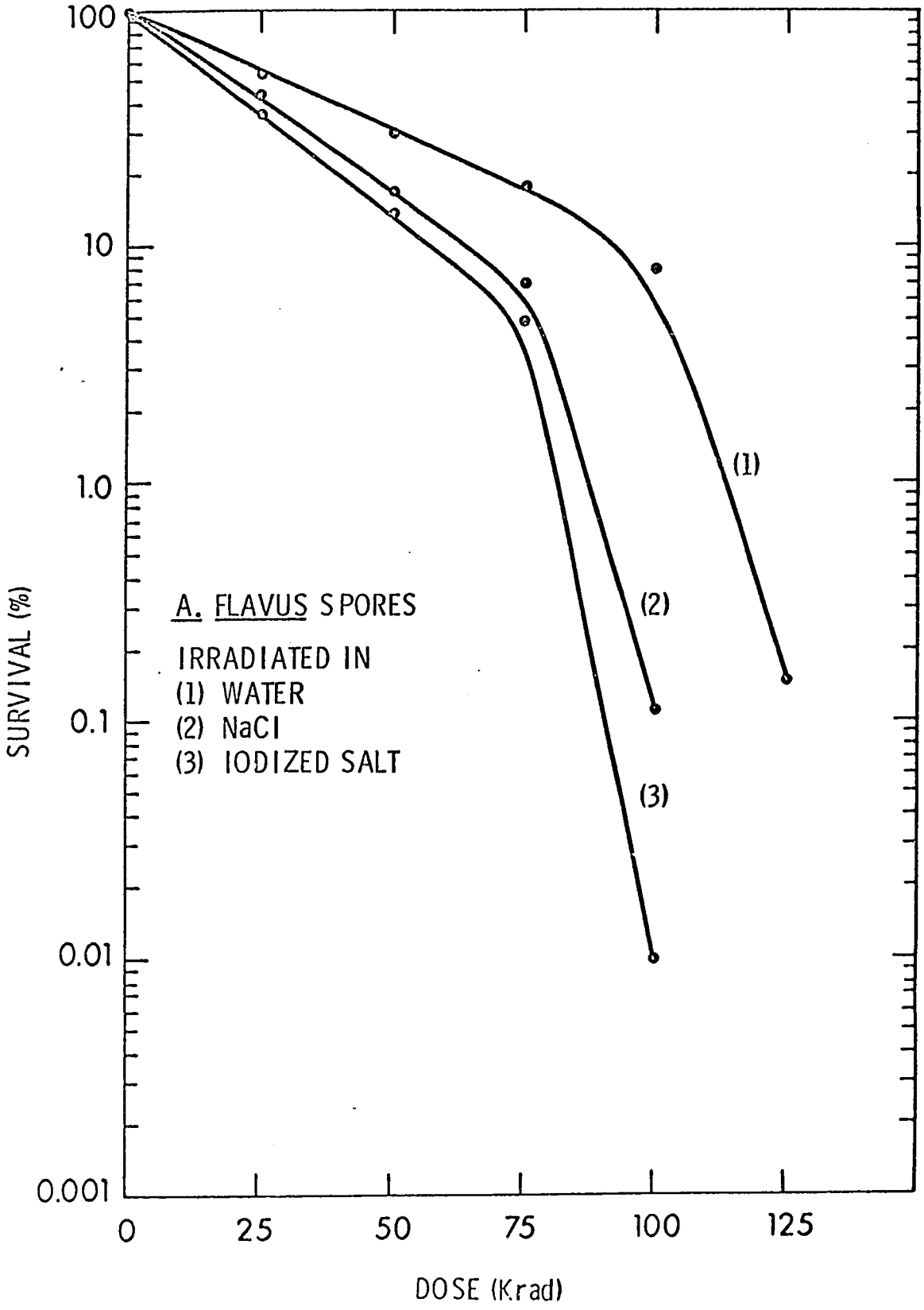

FIG. 5. ϒ-irradiation survival curves for spores of A.flavus, irradiated in a solution of NaCl or iodized salt.

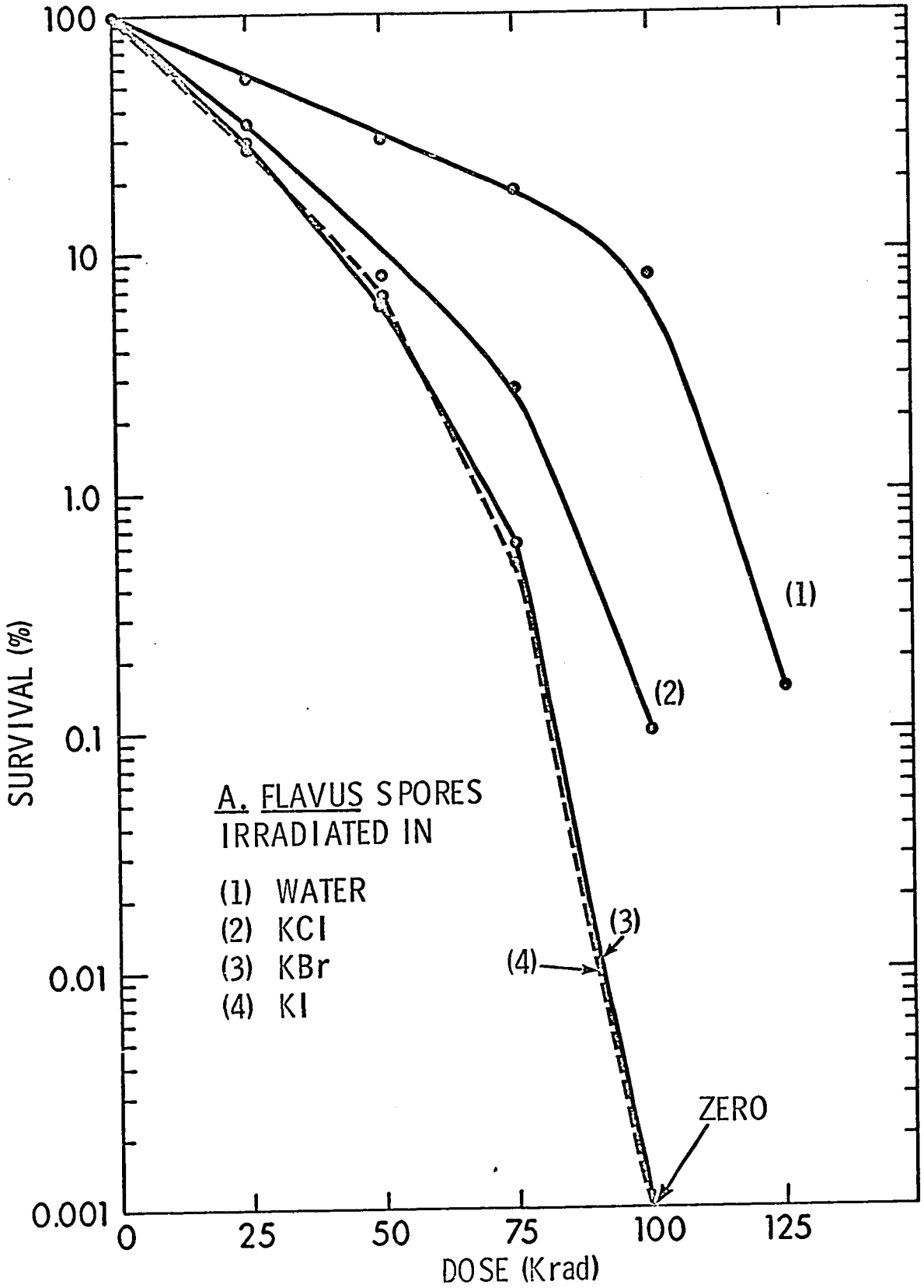

FIG. 6. γ-irradiation survival curves for spores of A. flavus, irradiated in a solution of KCl, KBr or KI.

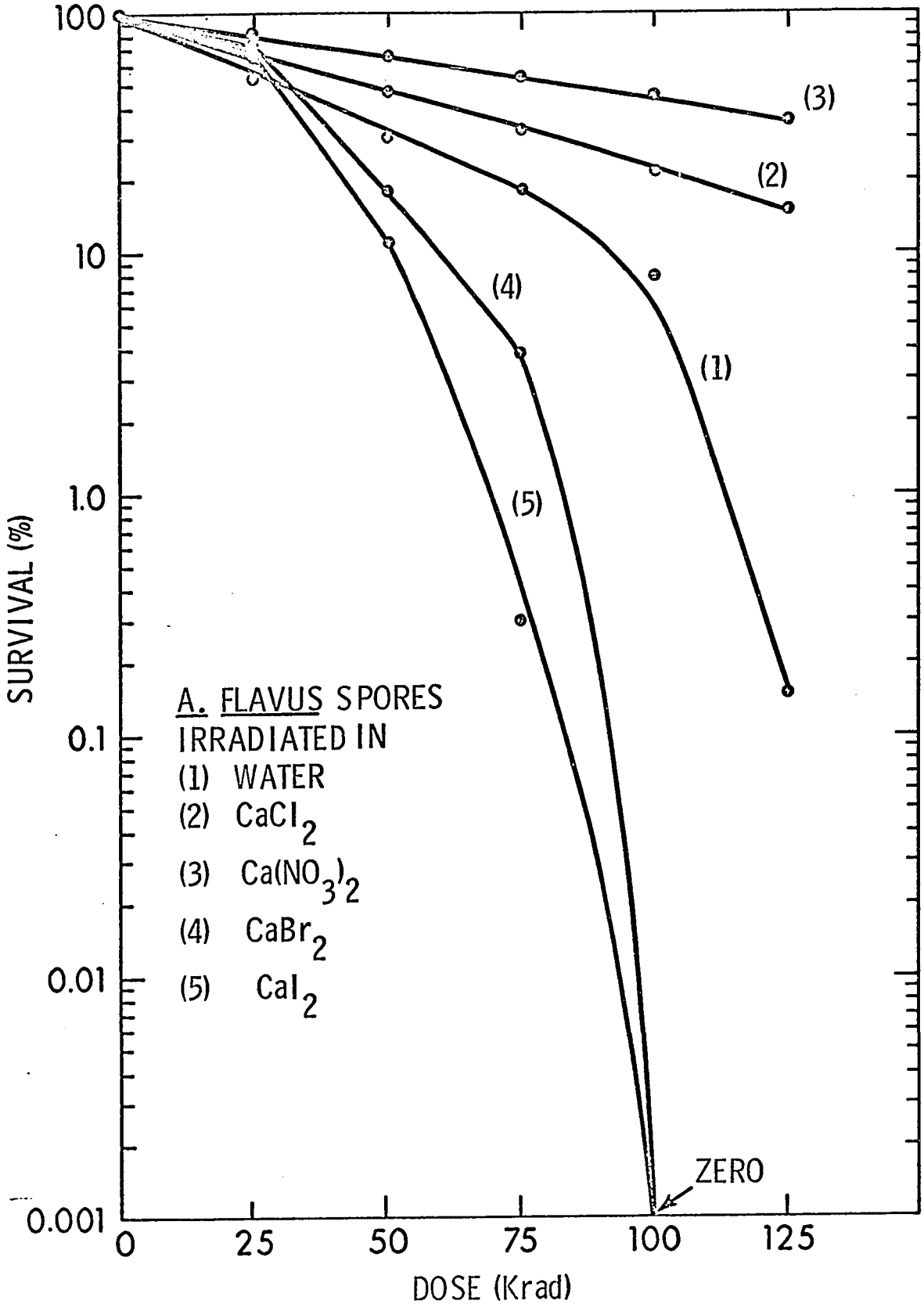

FIG. 7. ɣ-irradiation survival curves for spores of A. flavus, irradiated in a solution of $CaCl_2$, $CaBr_2$, CaI_2 or $Ca(NO_3)_2$.

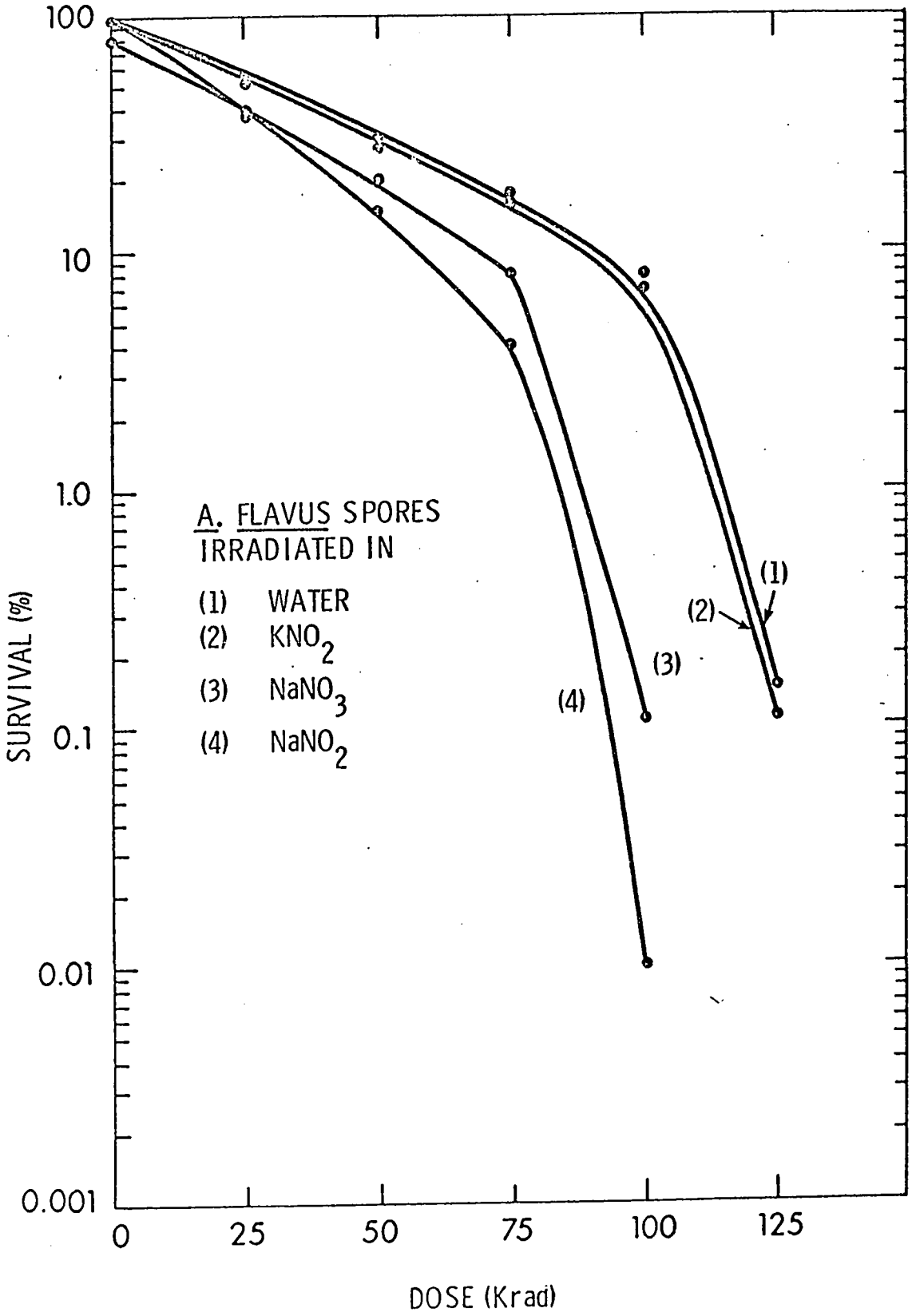

FIG. 8. ϒ-irradiation survival curves for spores of A. flavus, irradiated in a solution of KNO_2, $NaNO_2$, or NaO_3.

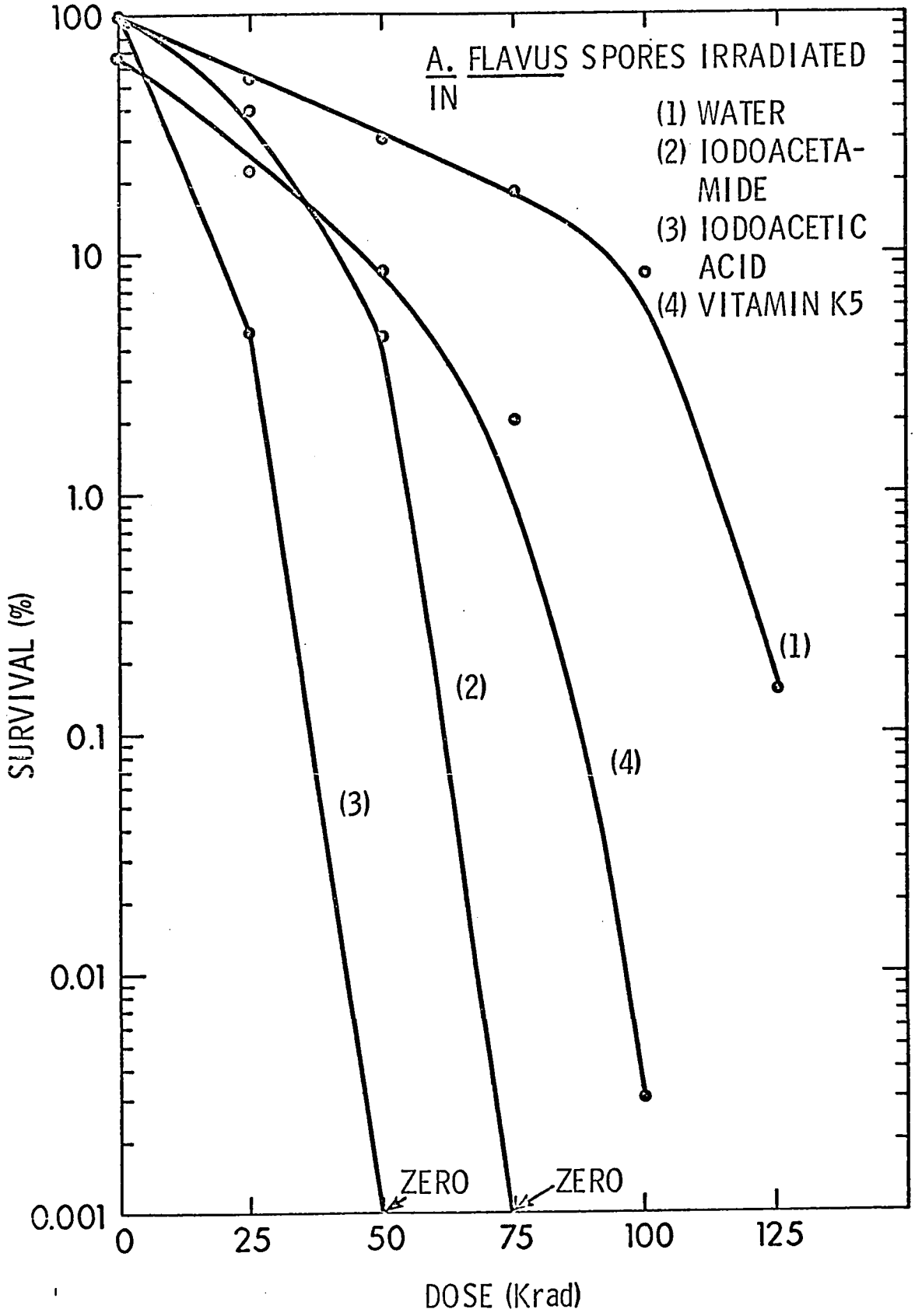

FIG. 9. γ-irradiation survival curves for spores of A. flavus, irradiated in a solution of iodoacetic acid, iodoacetamide or vitamin K_5.

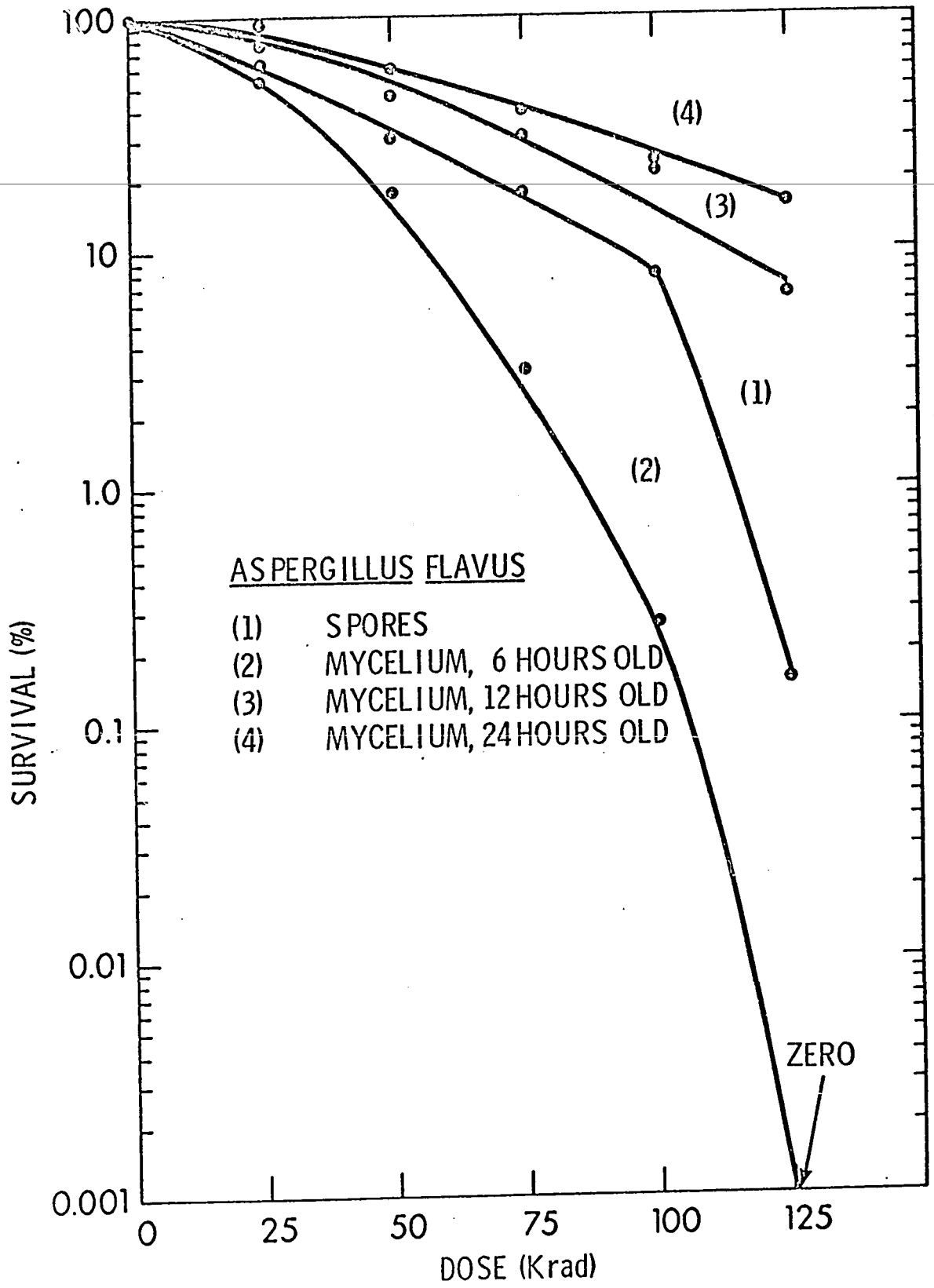

FIG. 10. γ-irradiation survival curves for spores of A. flavus, irradiated without prior incubation or irradiated after incubation for different periods of time.

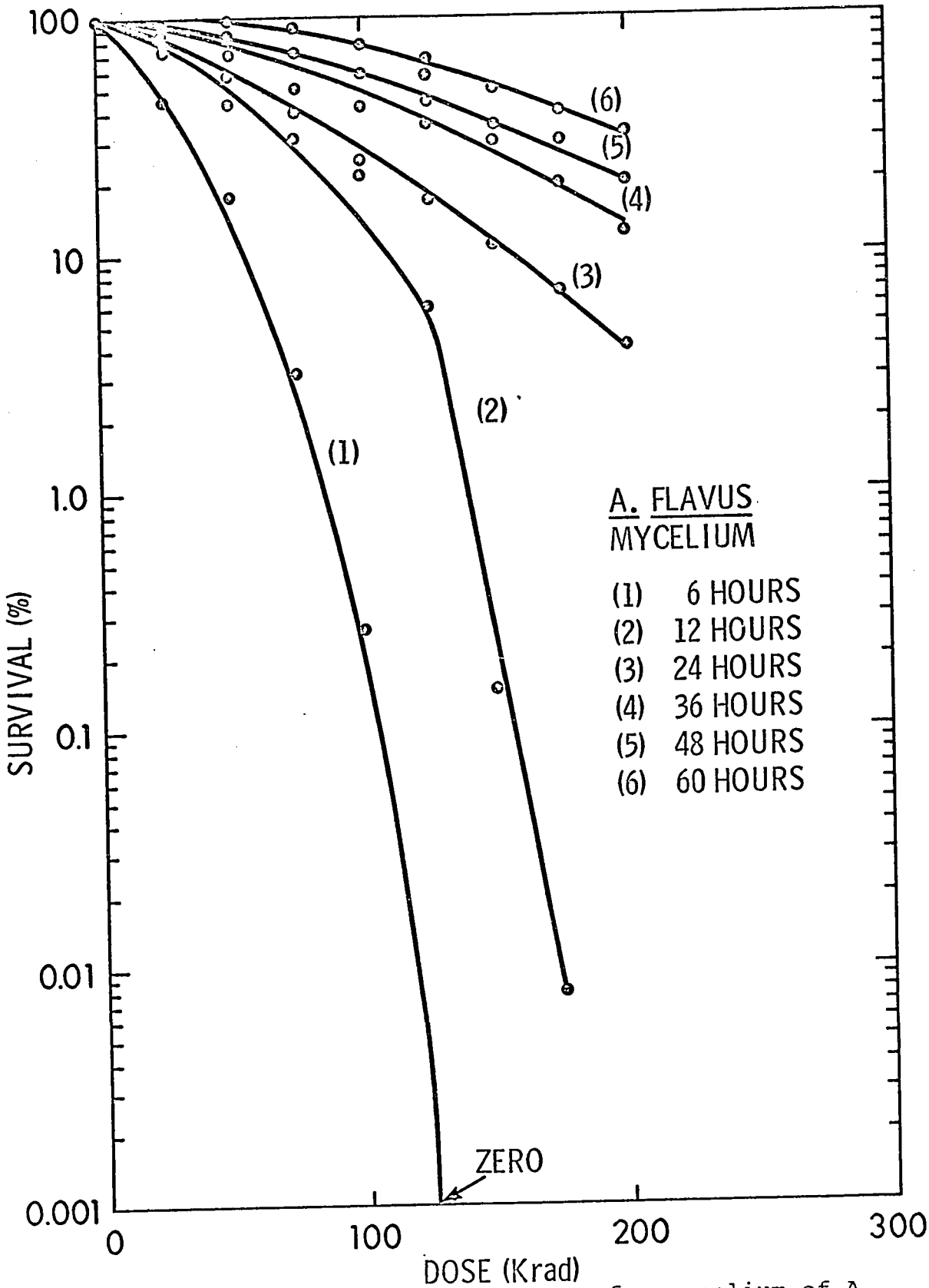

FIG. 11. γ-irradiation survival curves for mycelium of A. flavus, irradiated after incubation for different periods of time.

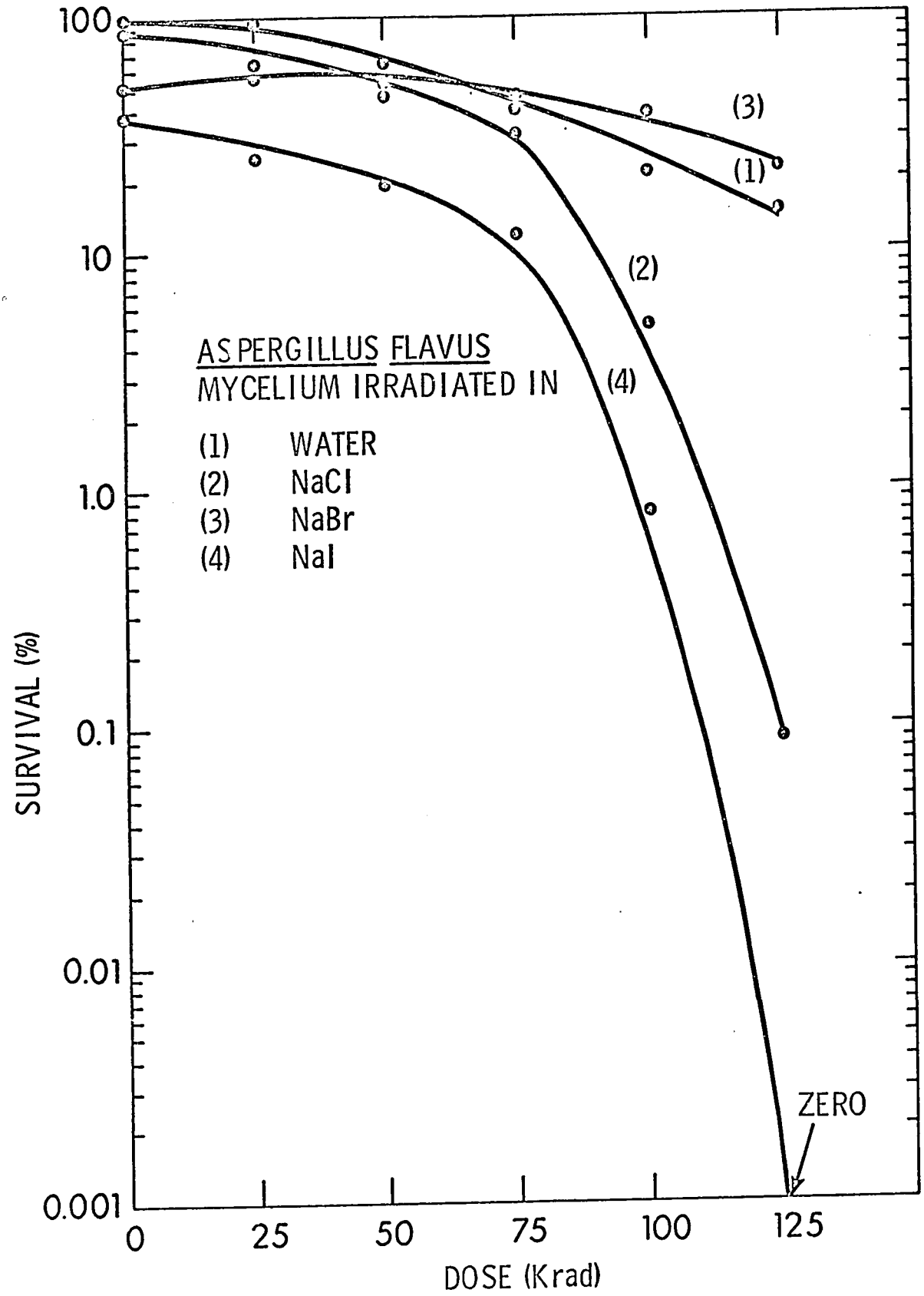

FIG.12. γ-irradiation survival curves for mycelium of A.flavus, irradiated in a solution of NaCl, NaBr or NaI.

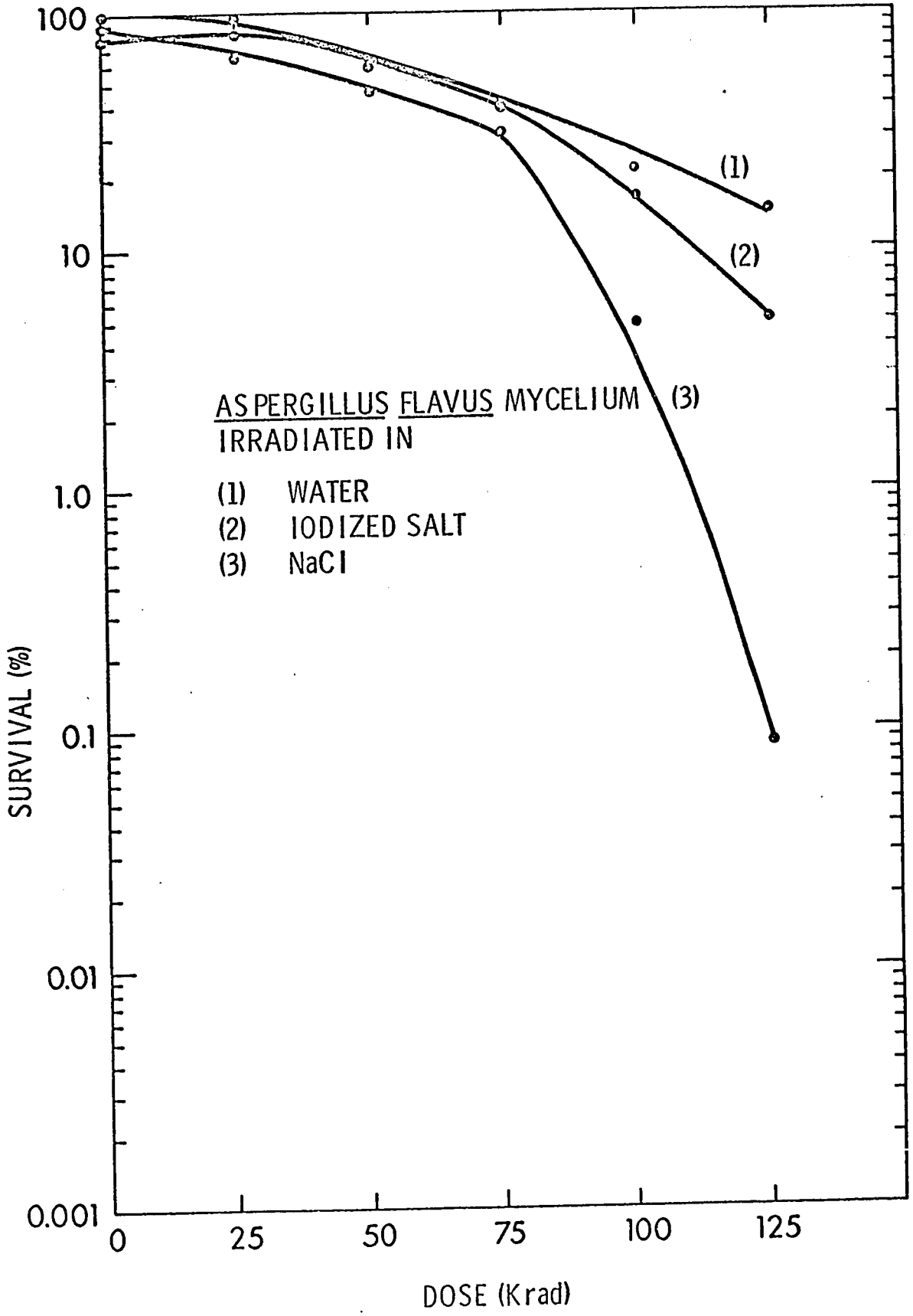

FIG.13. ɣ-irradiation survival curves for mycelium of A.flavus, irradiated in a solution of NaCl or iodized salt.

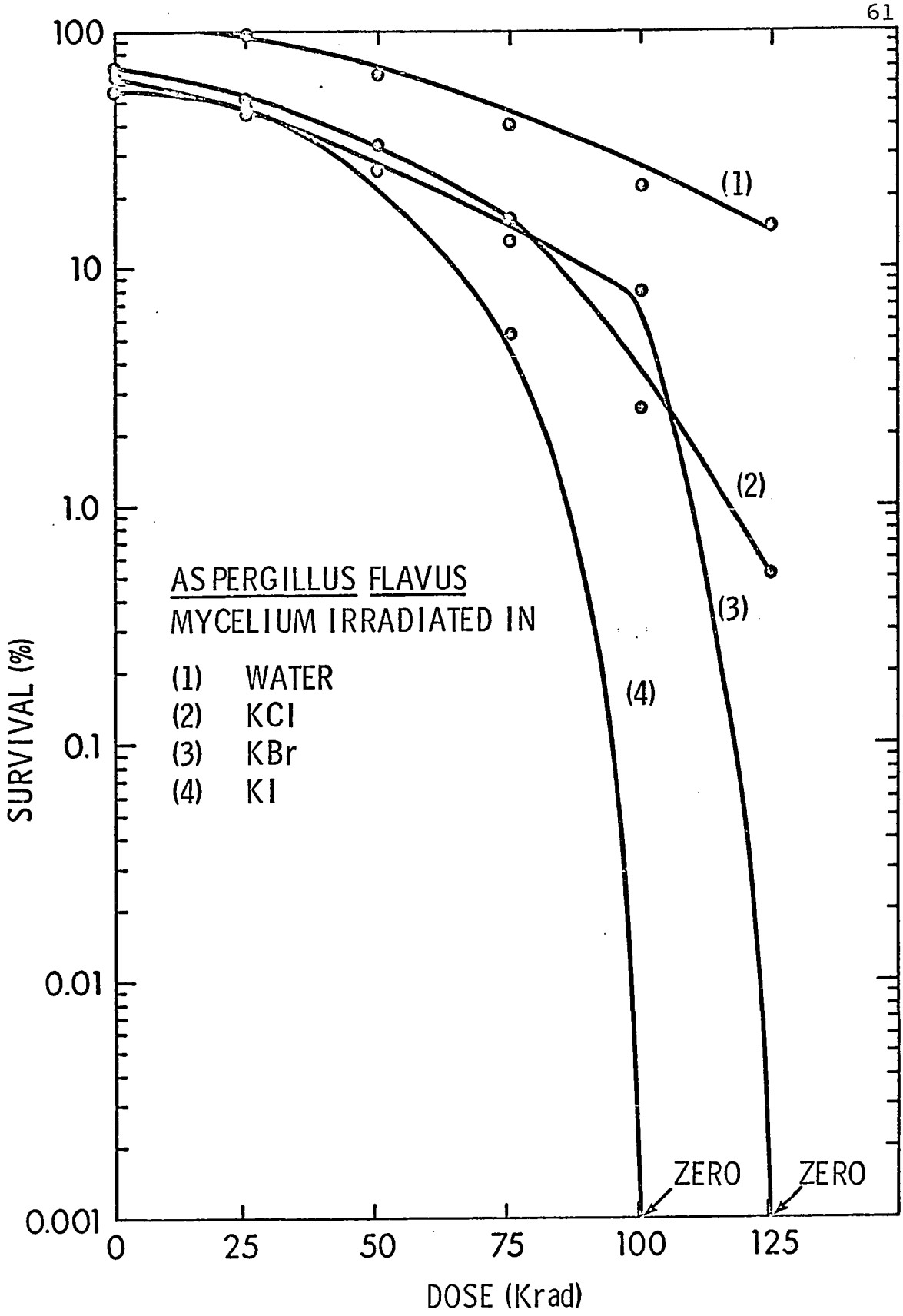

FIG.14. γ-irradiation survival curves for mycelium of A. flavus, irradiated in a solution of KCl, KBr or KI.

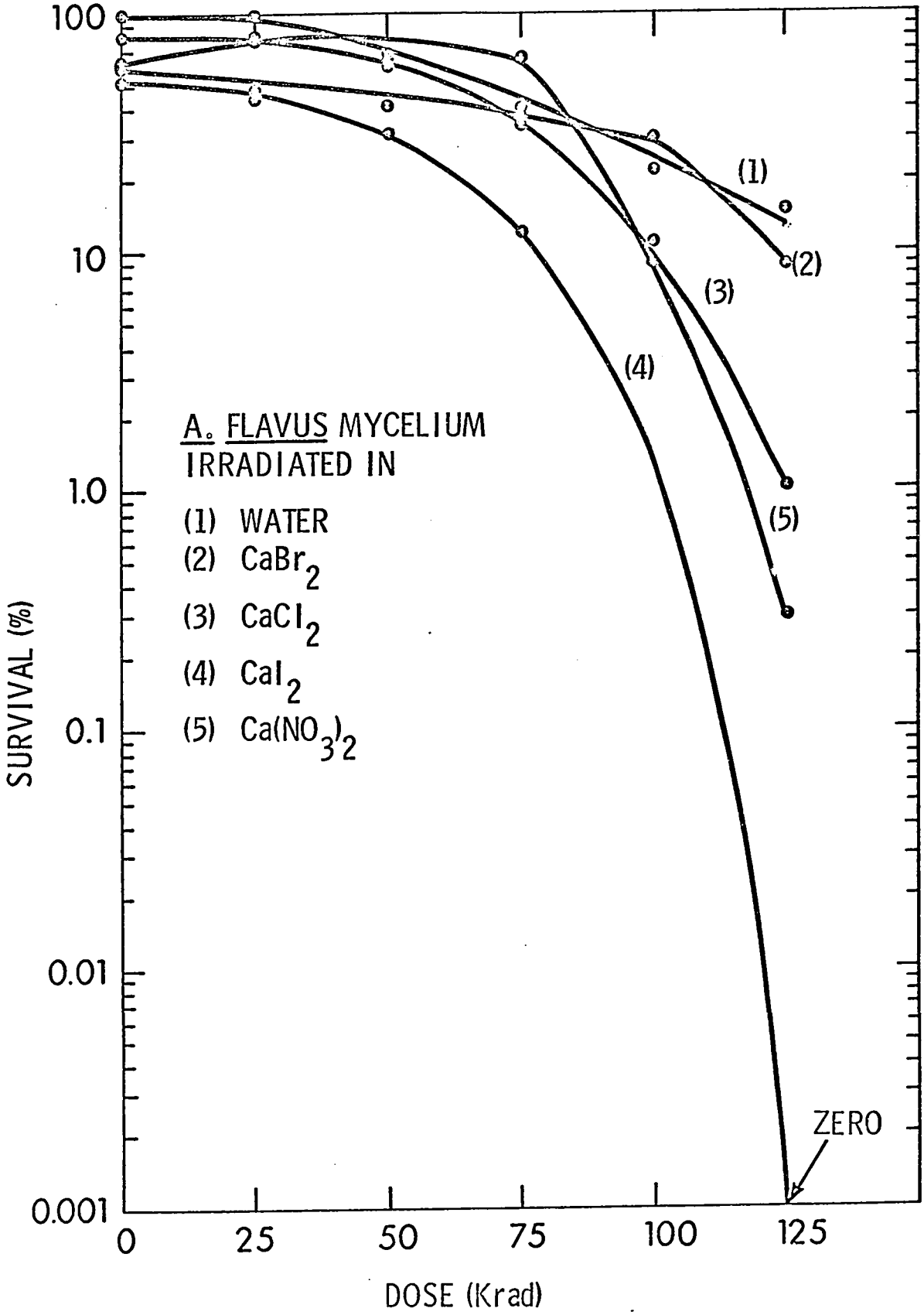

FIG.15. ɣ-irradiation survival curves for mycelium of A. flavus, irradiated in a solution of $CaCl_2$, $CaBr_2$, CaI_2 or $Ca(NO_3)_2$.

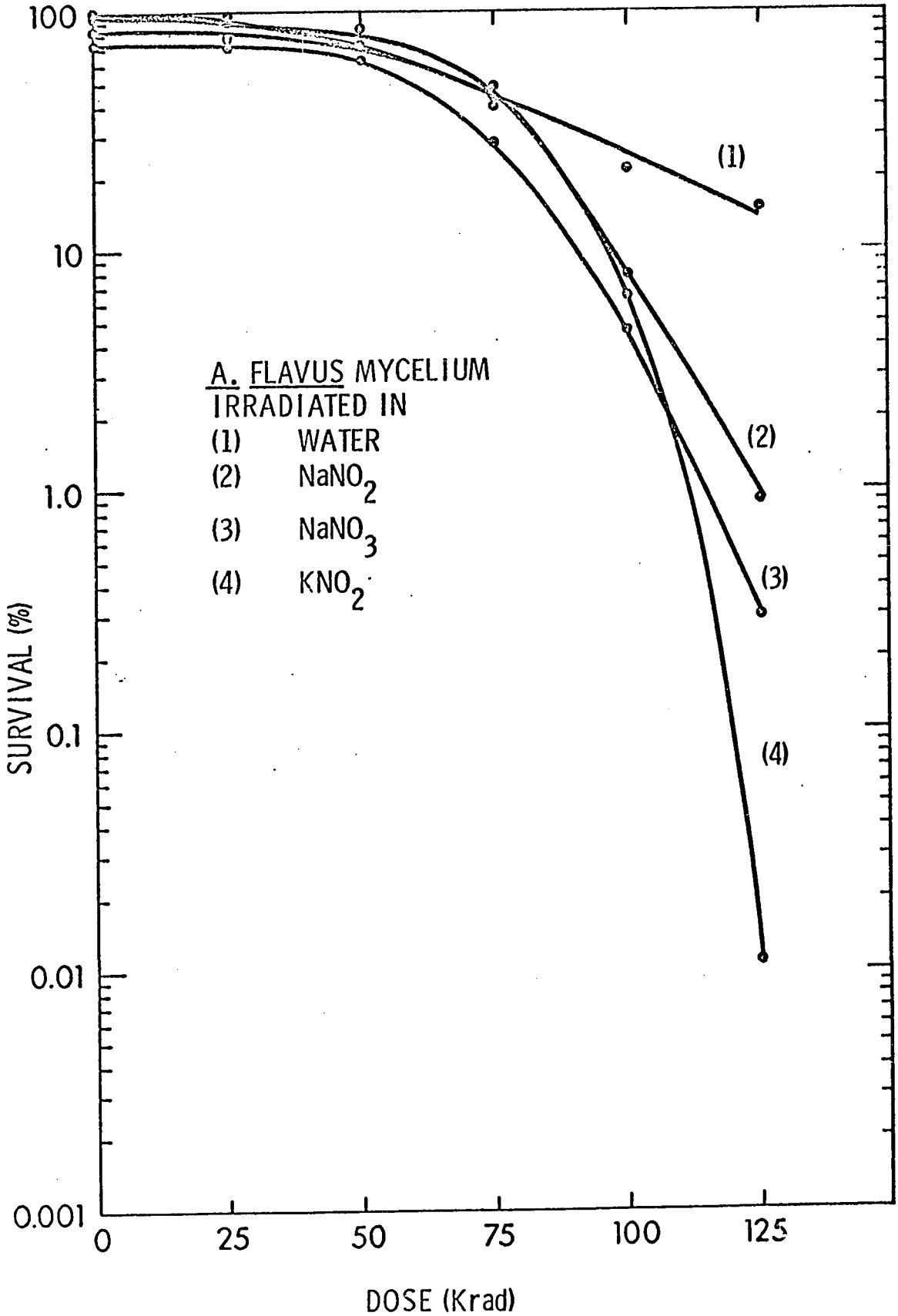

FIG.16. γ-irradiation survival curves for mycelium of A. flavus, irradiated in a solution of $NaNO_2$, $NaNO_3$ or KNO_2.

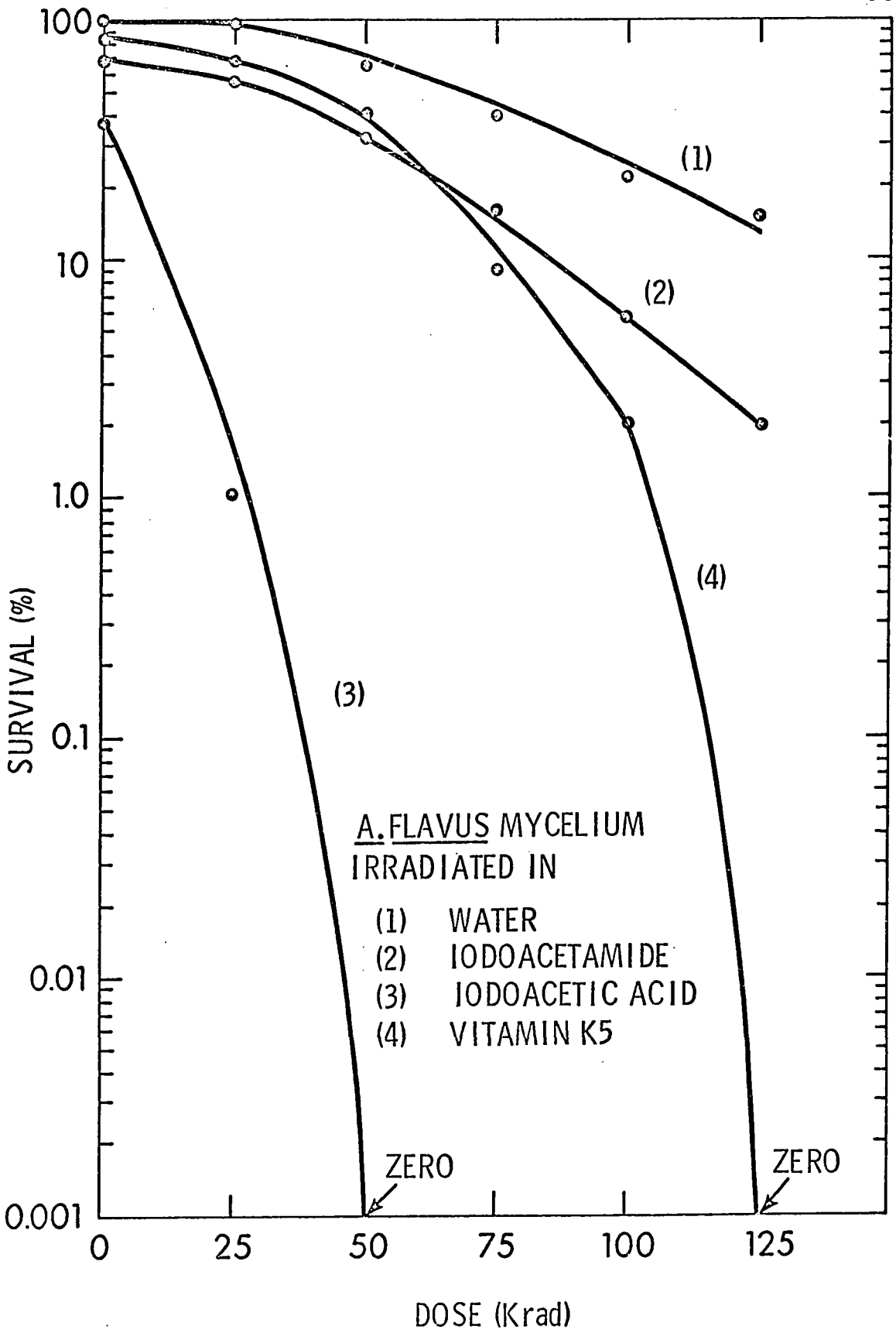

FIG. 17. γ-irradiation survival curves for mycelium of A. flavus, irradiated in a solution of iodoacetic acid, iodoacetamide or vitamin K_5.

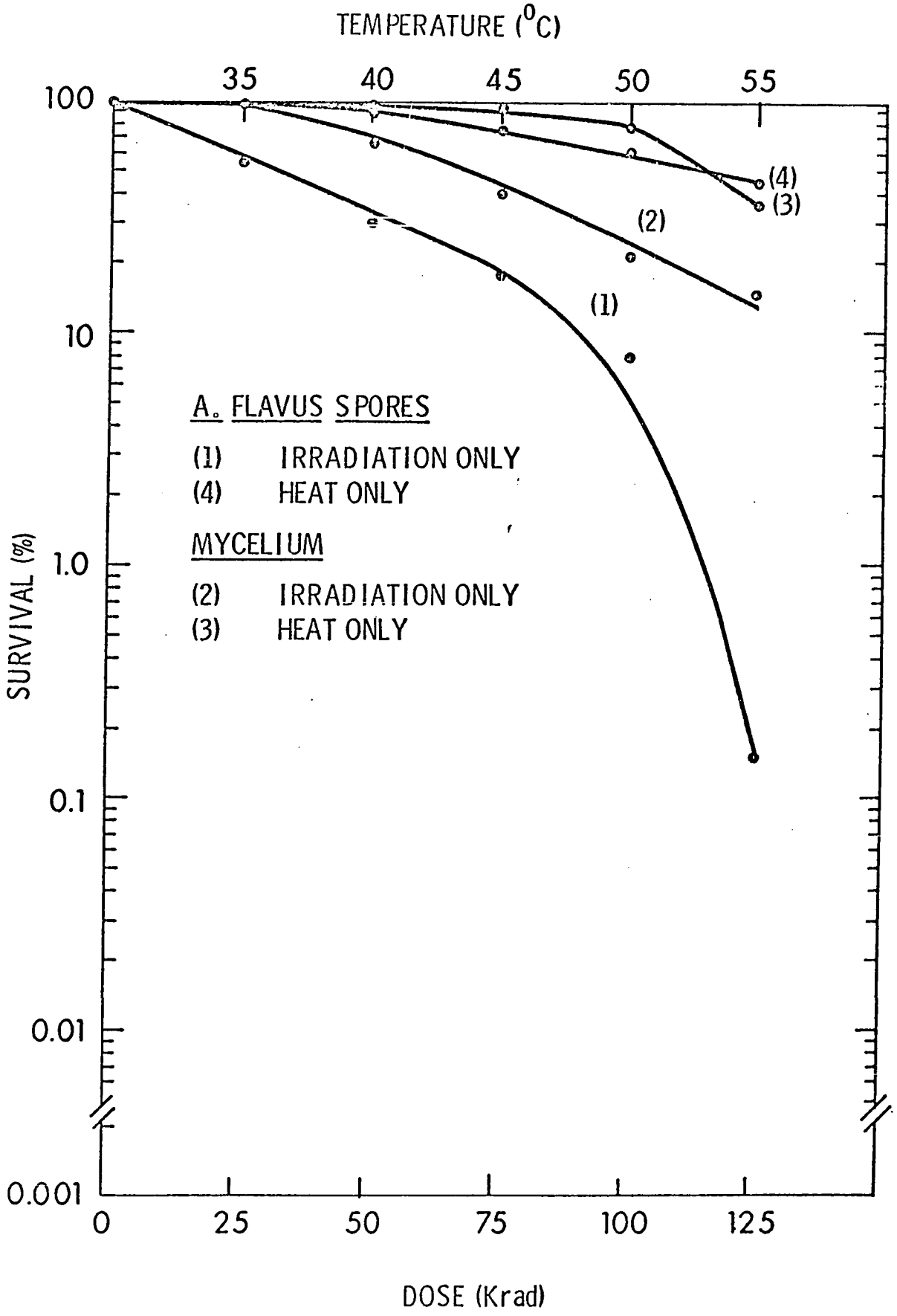

FIG.18. γ-irradiation and temperature sensitivities of spores and mycelium of A.flavus.

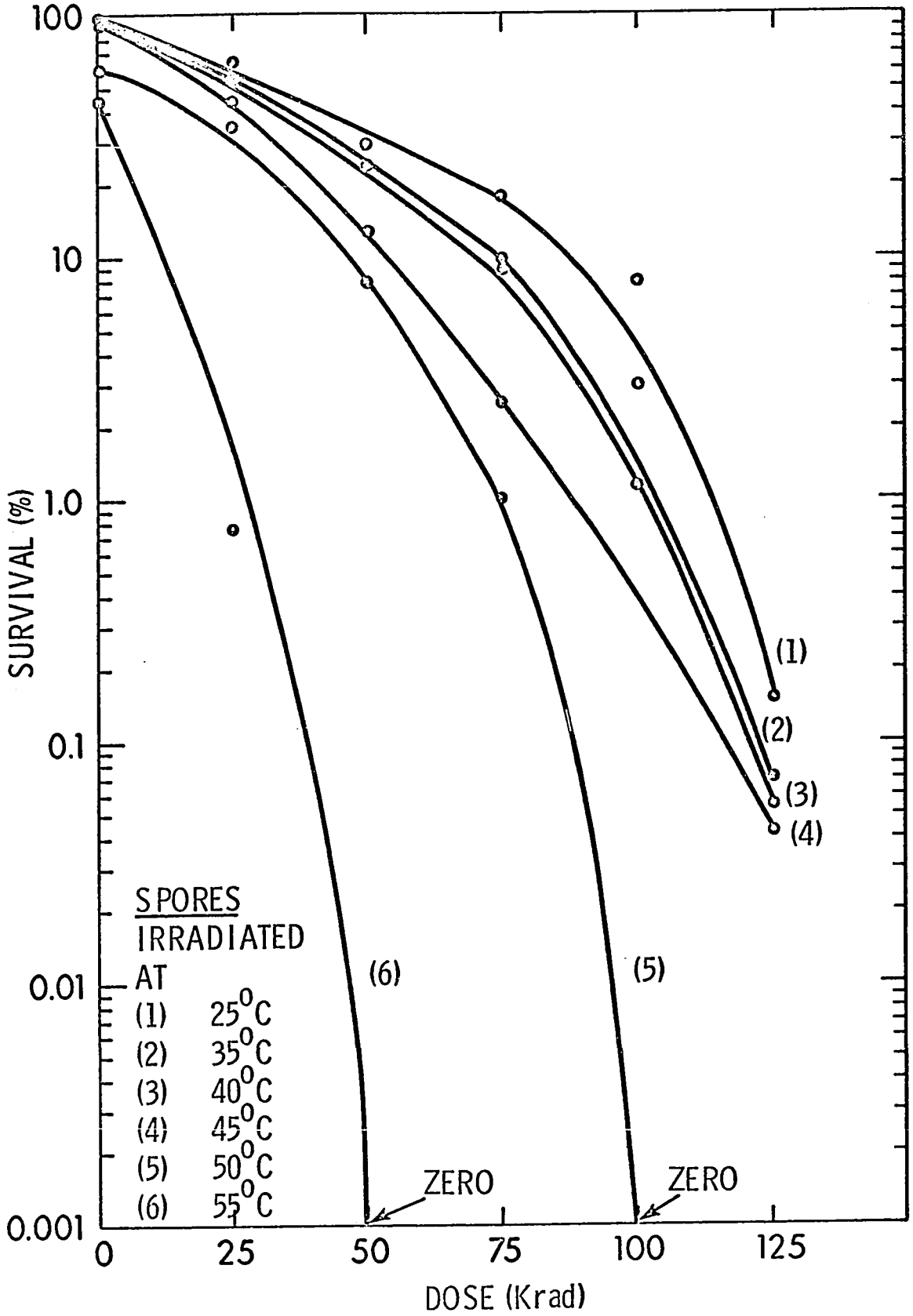

FIG.19. Survival curves for spores of A.flavus, exposed to different temperatures during irradiation.

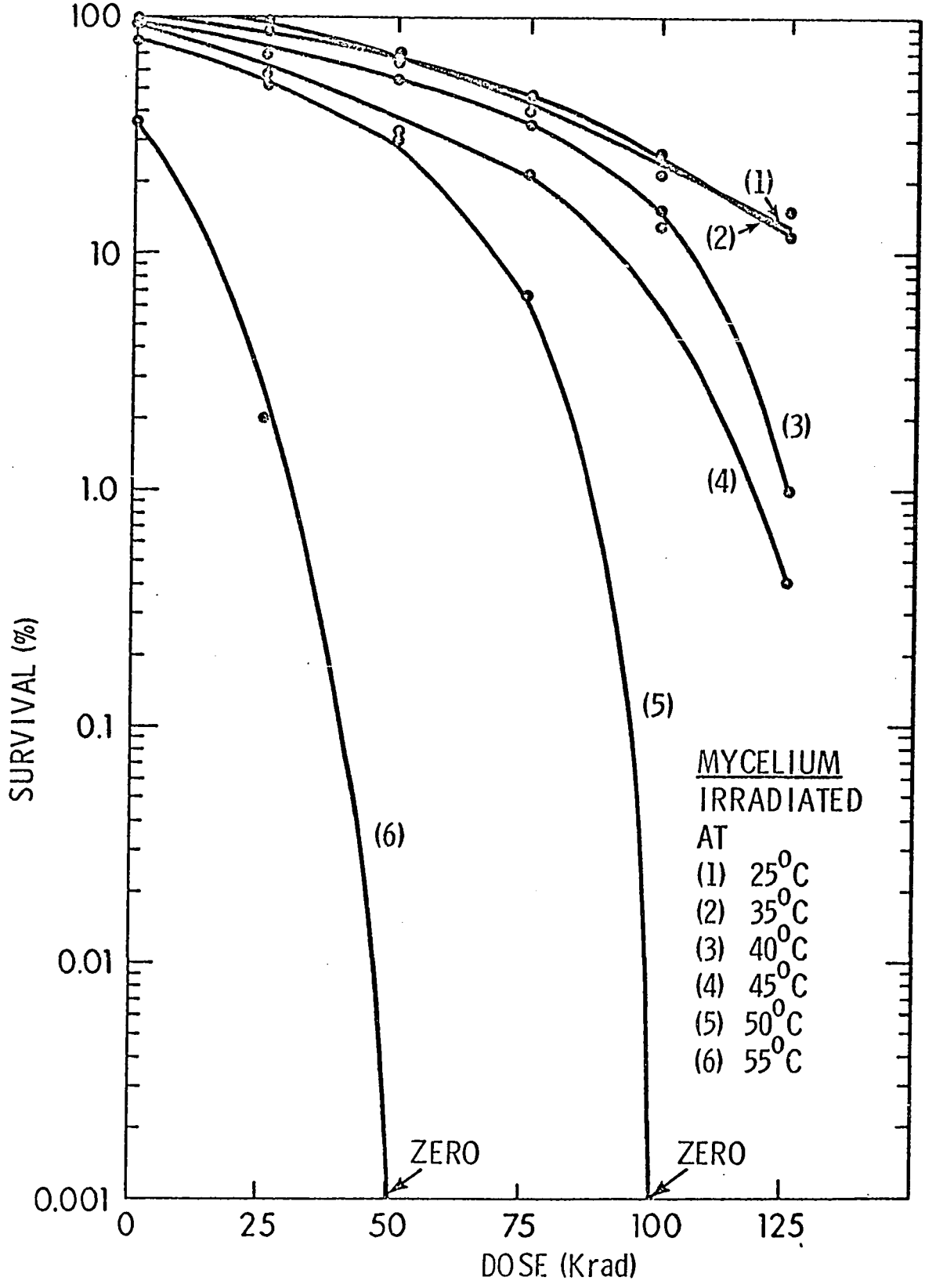

FIG.20. Survival curves for mycelium of A.flavus, exposed to different temperatures during irradiation.

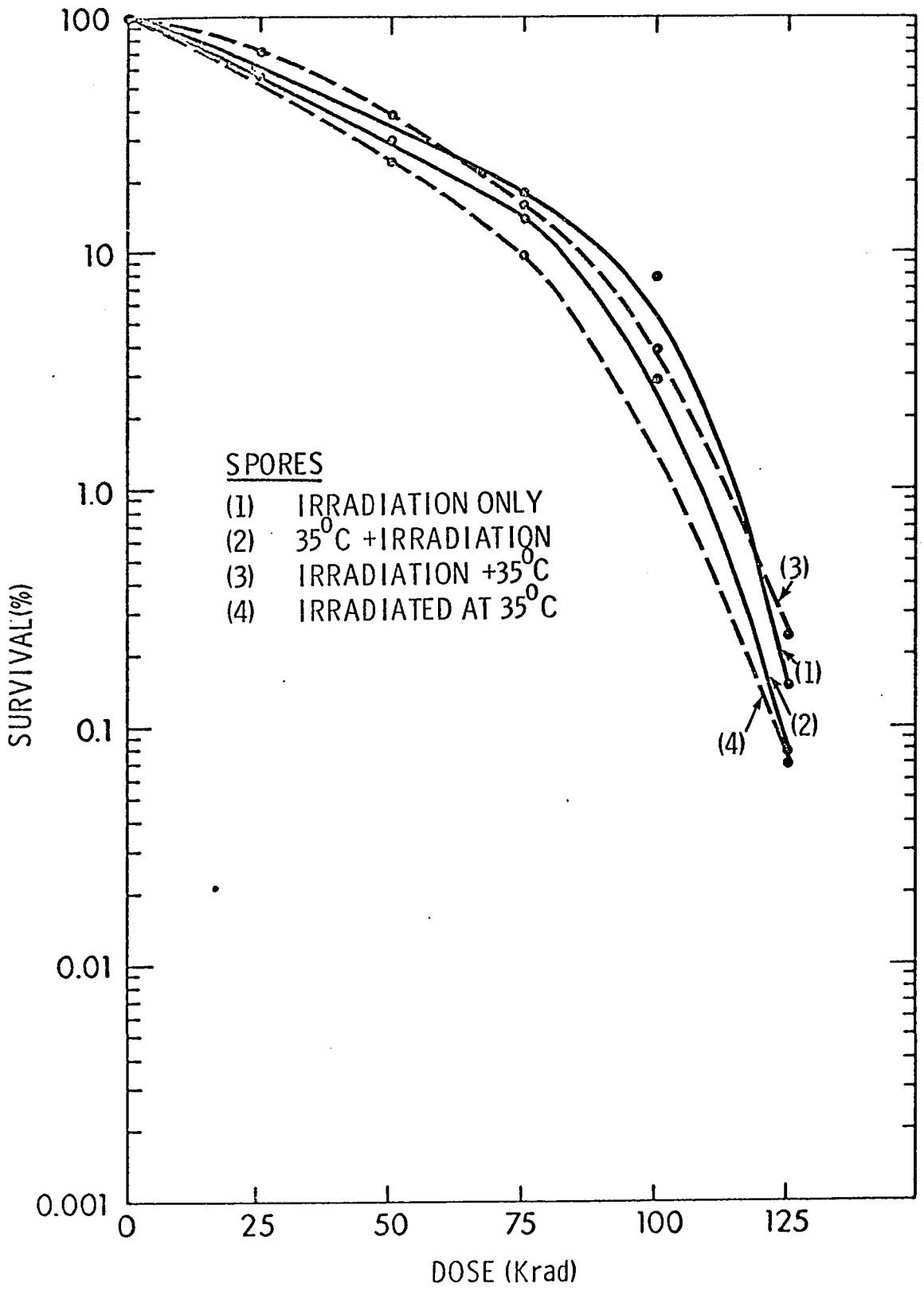

FIG. 21. The effect of different sequences of heat treatment at 35°C and different doses of γ-irradiation on the survival of spores of A. flavus.

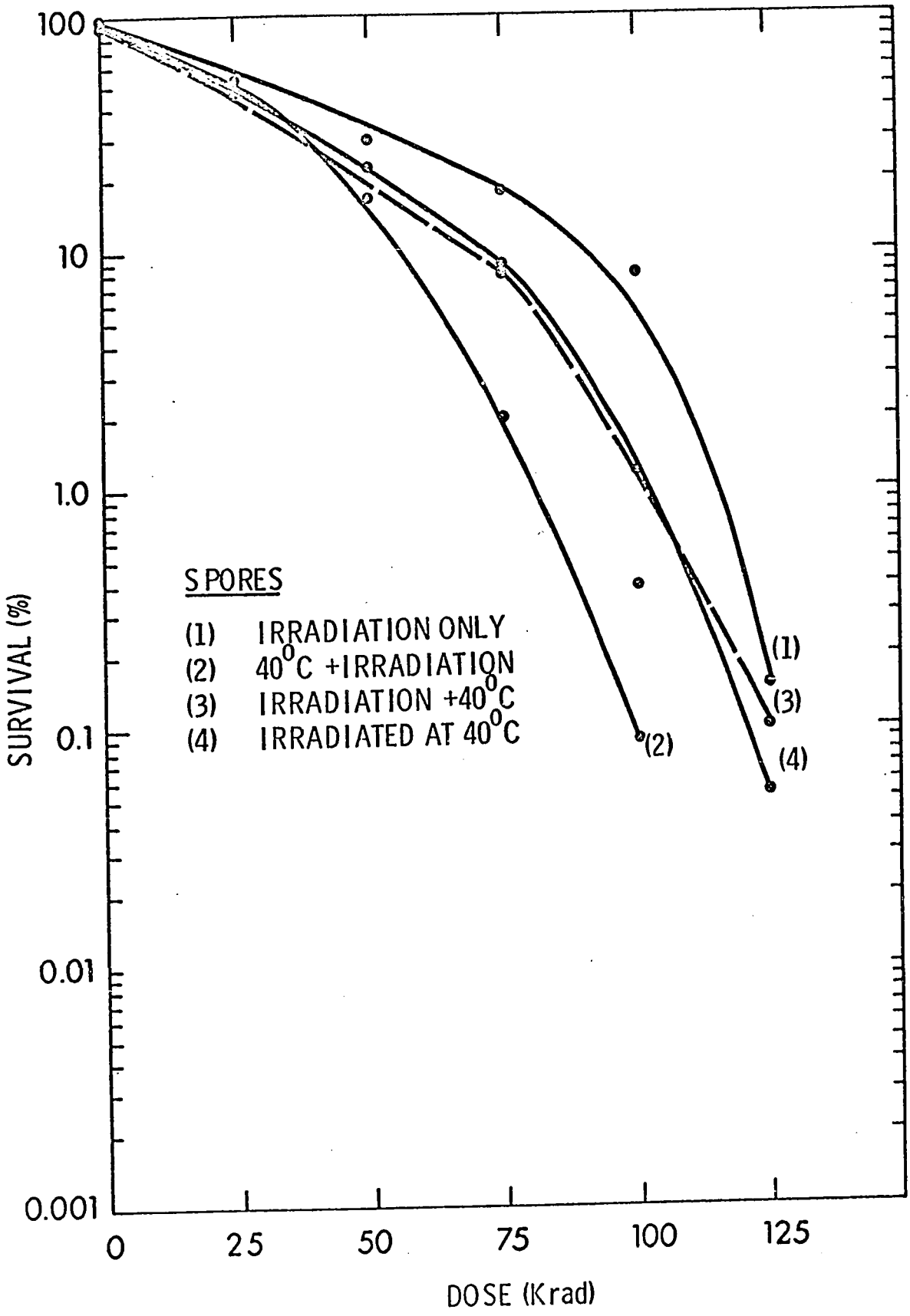

FIG.22. The effect of different sequences of heat treatment at 40°C and different doses of γ-irradiation on the survival of spores of A.flavus

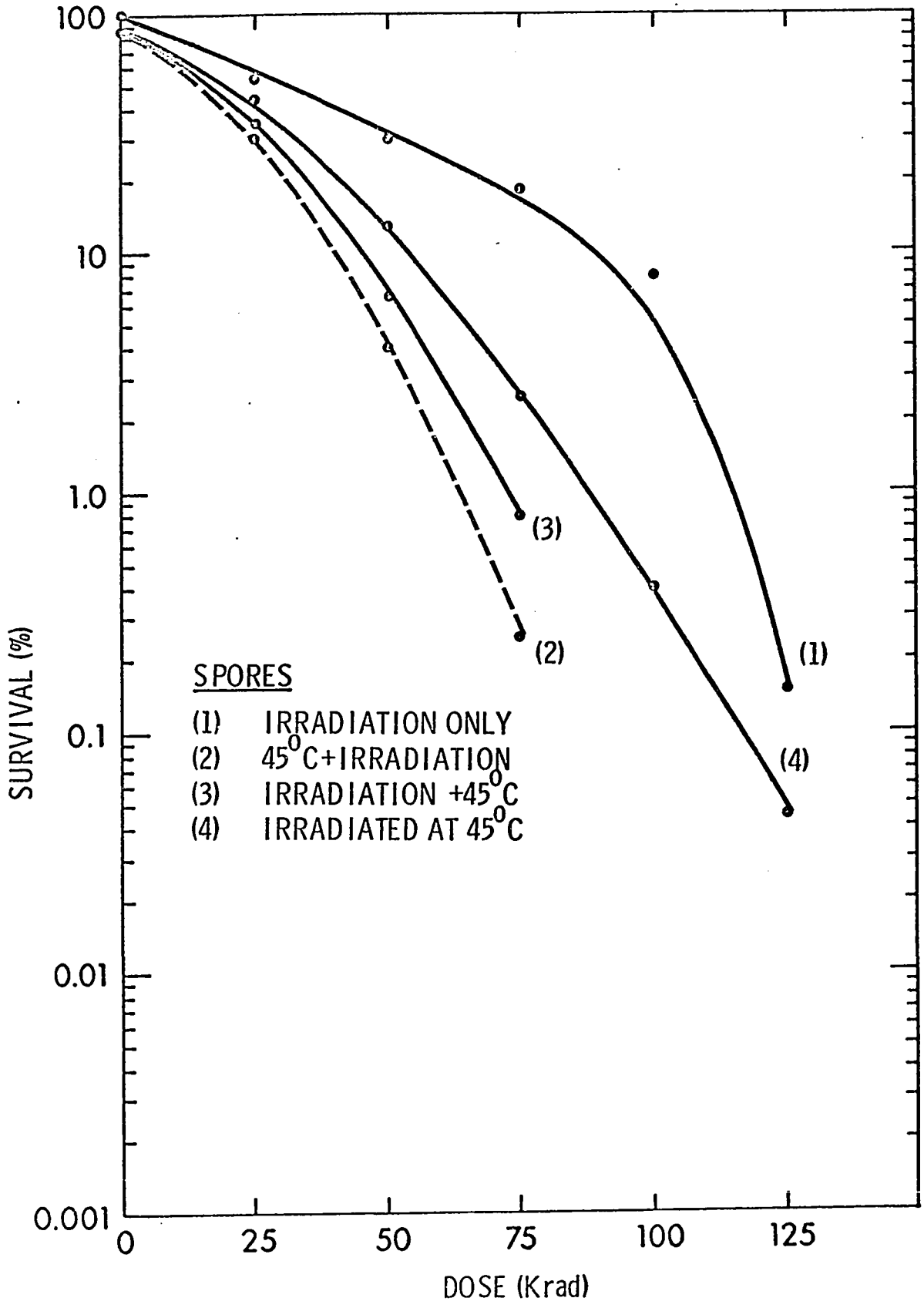

FIG. 23. The effect of different sequences of heat treatment at 45°C and different doses of γ-irradiation on the survival of spores of A. flavus.

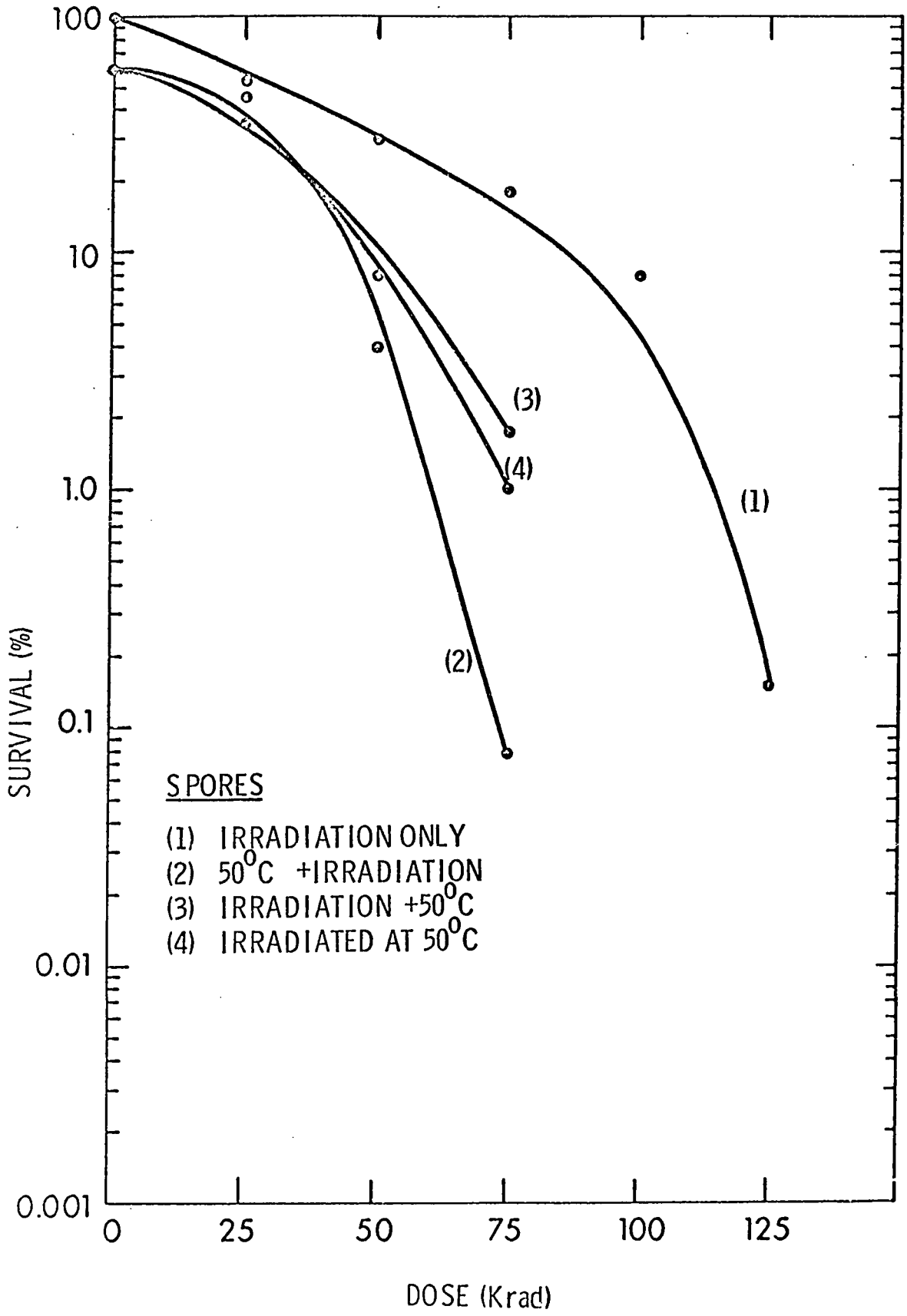

FIG.24. The effect of different sequences of heat treatment at 50°C and different doses of γ-irradiation on the survival of spores of A.flavus.

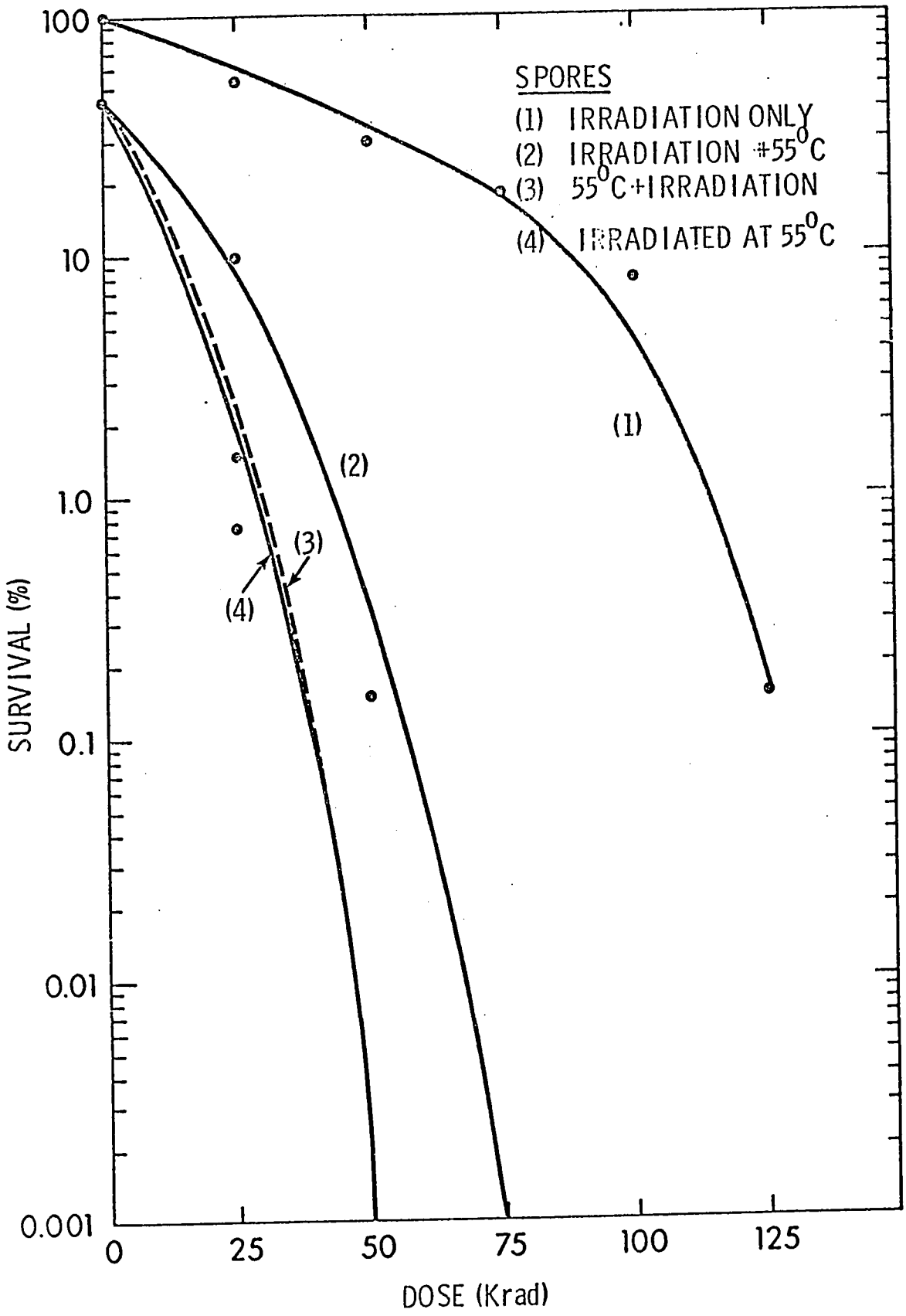

FIG.25. The effect of different sequences of heat treatment at 55°C and different doses of ϑ-irradiation on the survival of spores of A.flavus.

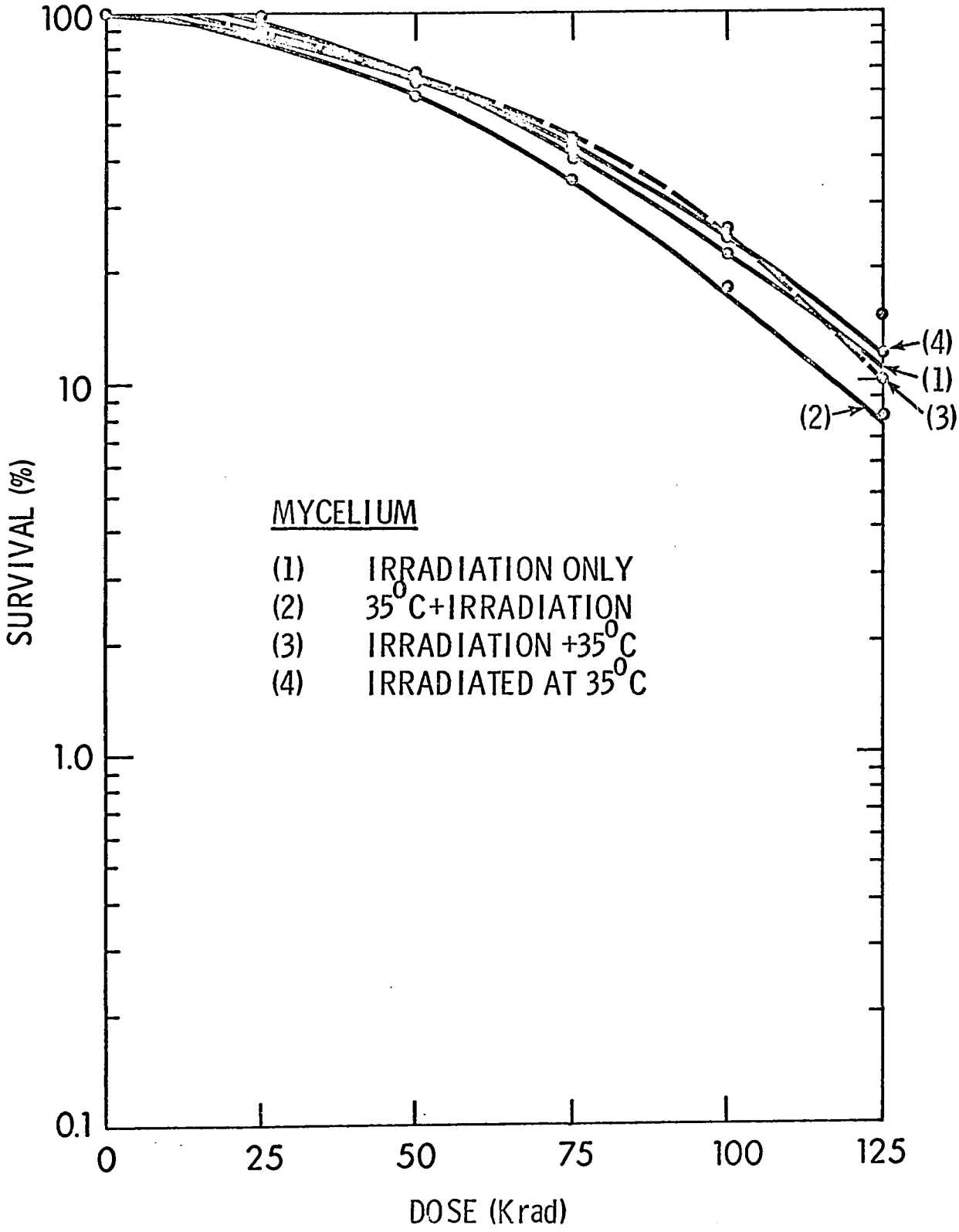

FIG. 26. The effect of different sequences of heat treatment at 35°C and different doses of γ-irradiation on the survival of mycelium of A. flavus.

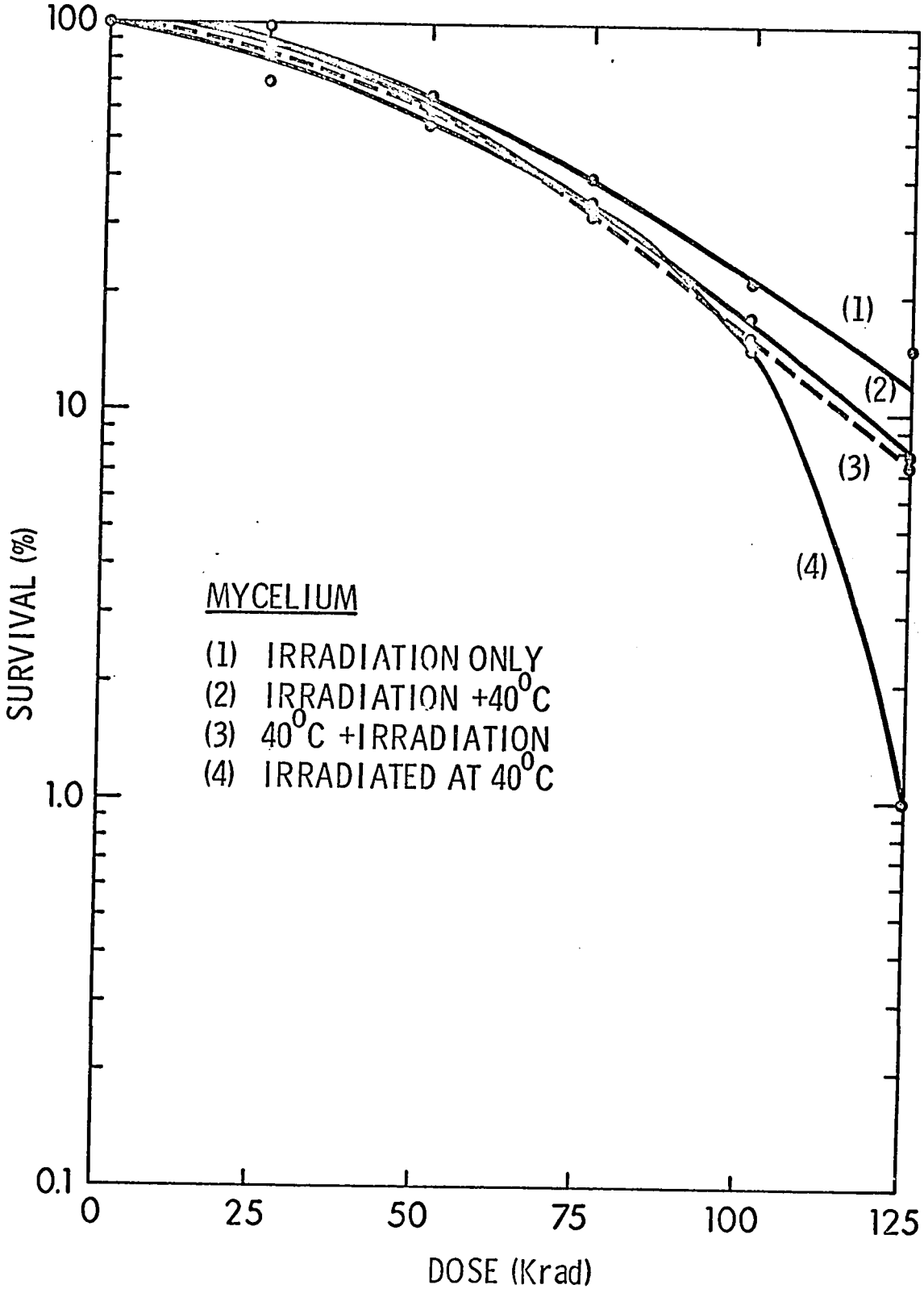

FIG.27. The effect of different sequences of heat treatment at 40°C and different doses of γ-irradiation on the survival of mycelium of A. flavus.

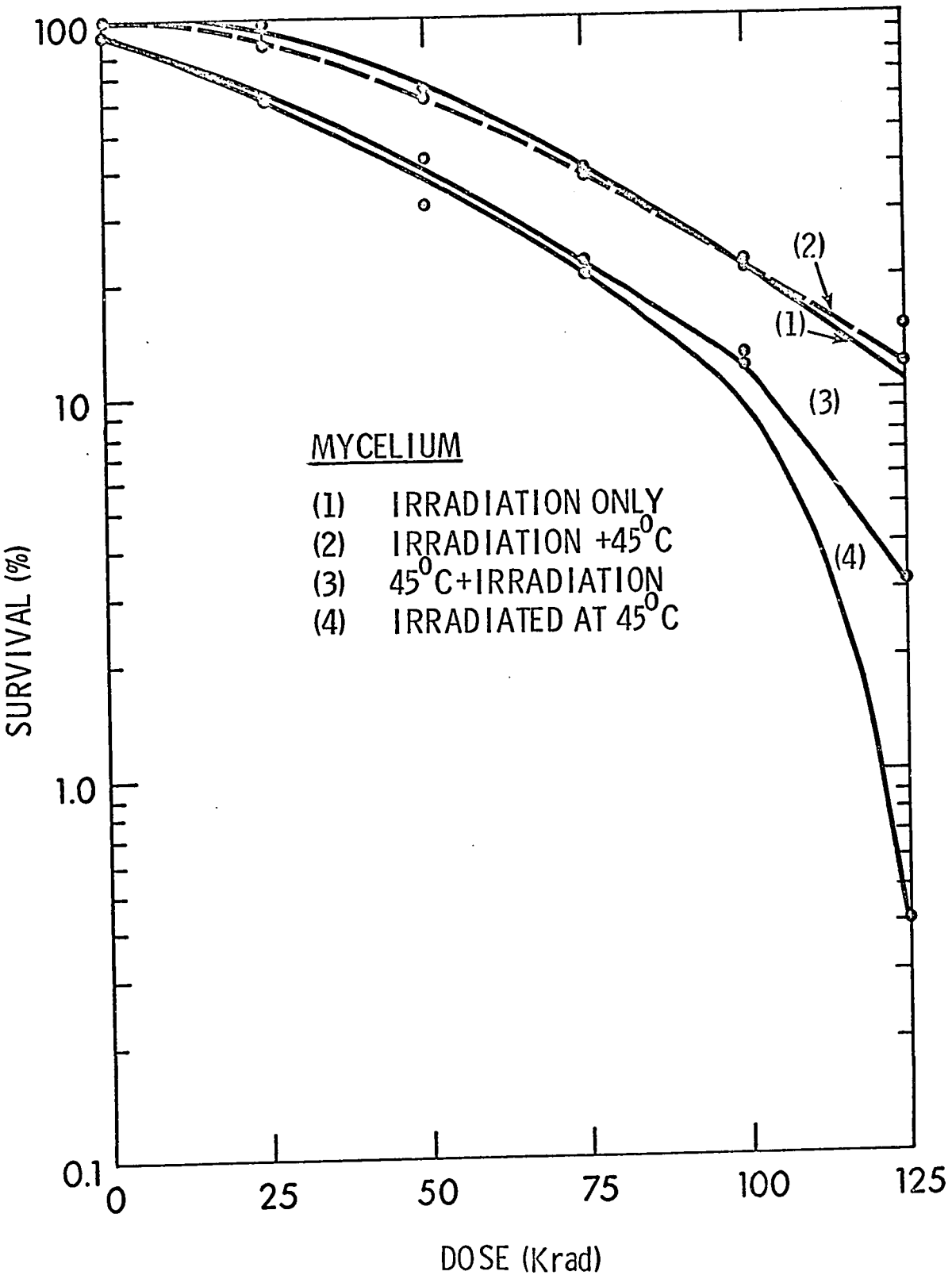

FIG.28. The effect of different sequences of heat treatment at 45°C and different doses of γ-irradiation on the survival of mycelium of A.flavus.

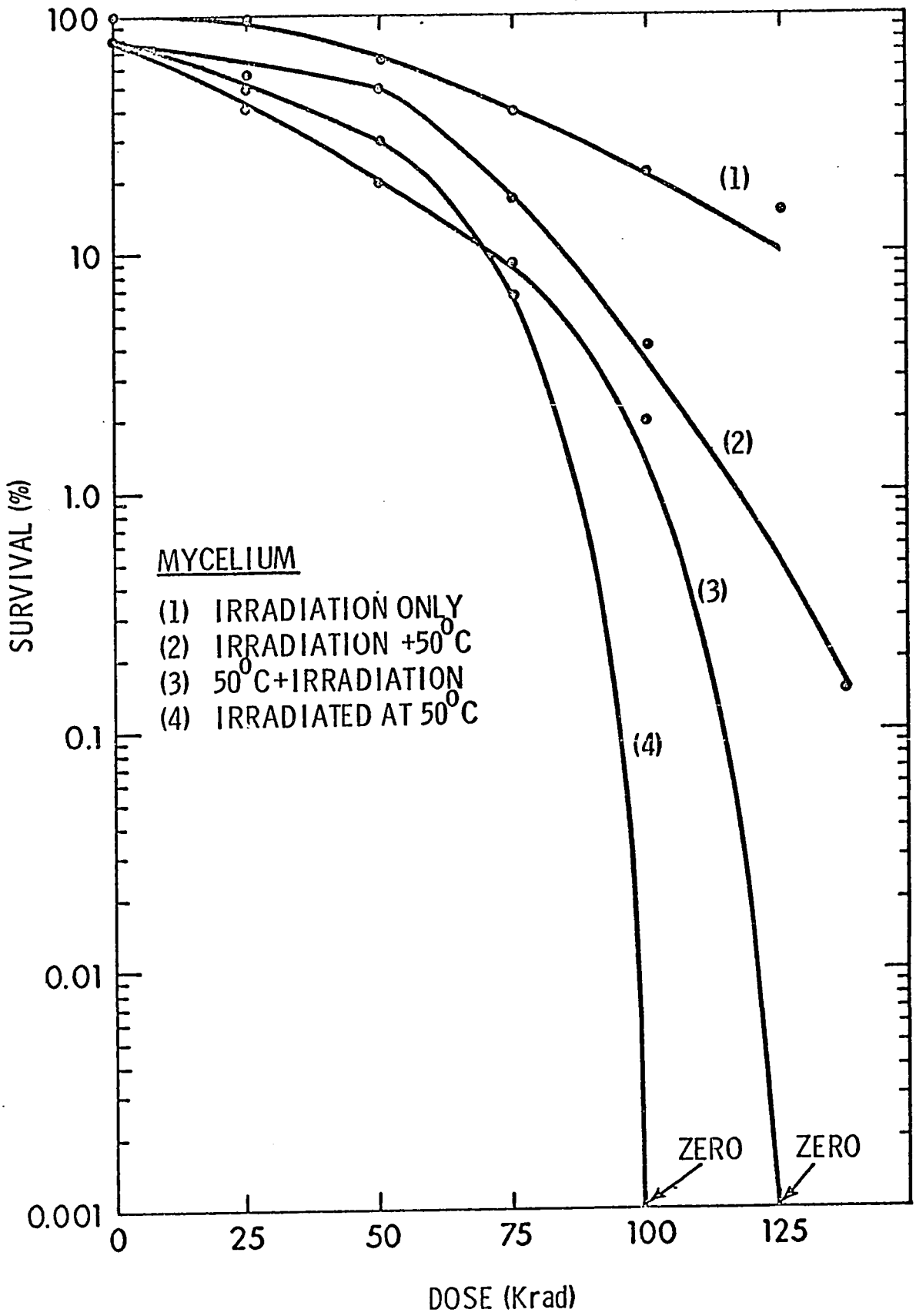

FIG. 29. The effect of different sequences of heat treatment of 50°C and different doses of γ-irradiation on the survival of mycelium of A. flavus.

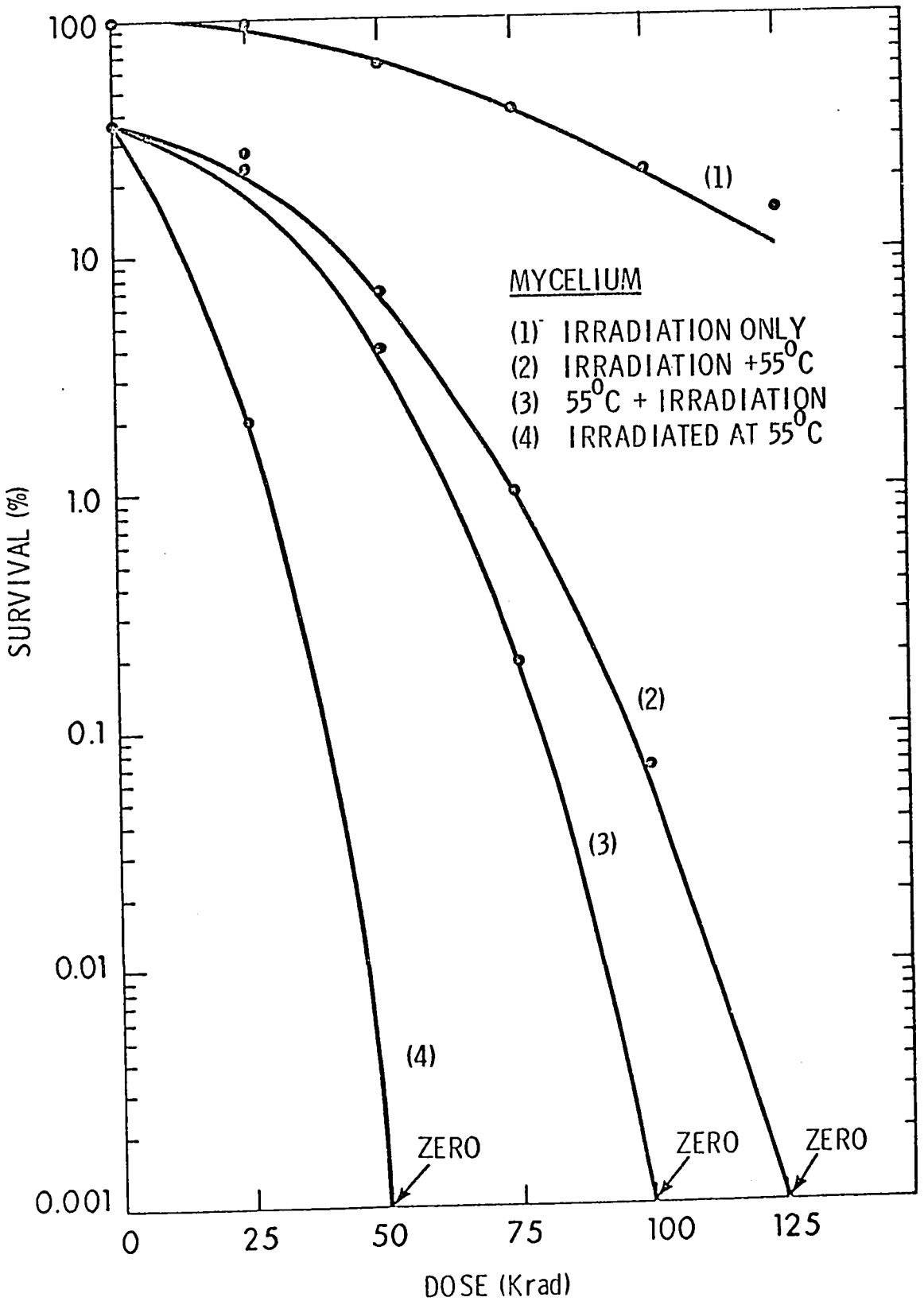

FIG. 30. The effect of different sequences of heat treatment at 55°C and different doses of γ-irradiation on the survival of mycelium of A.flavus.

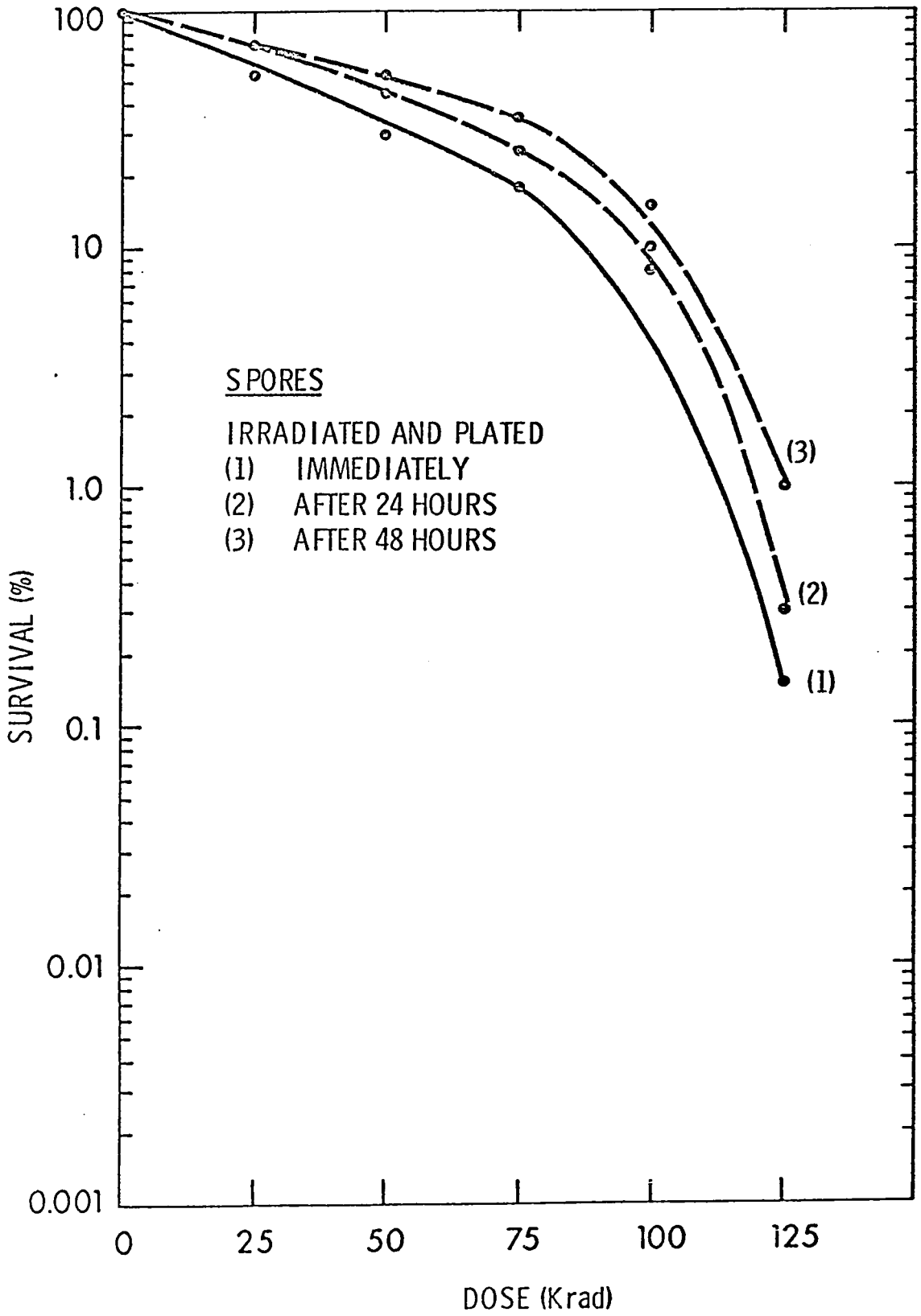

FIG. 31. Survival of spores of A. flavus as influenced by two different intervals of time between irradiation and plating on Czapek agar medium.

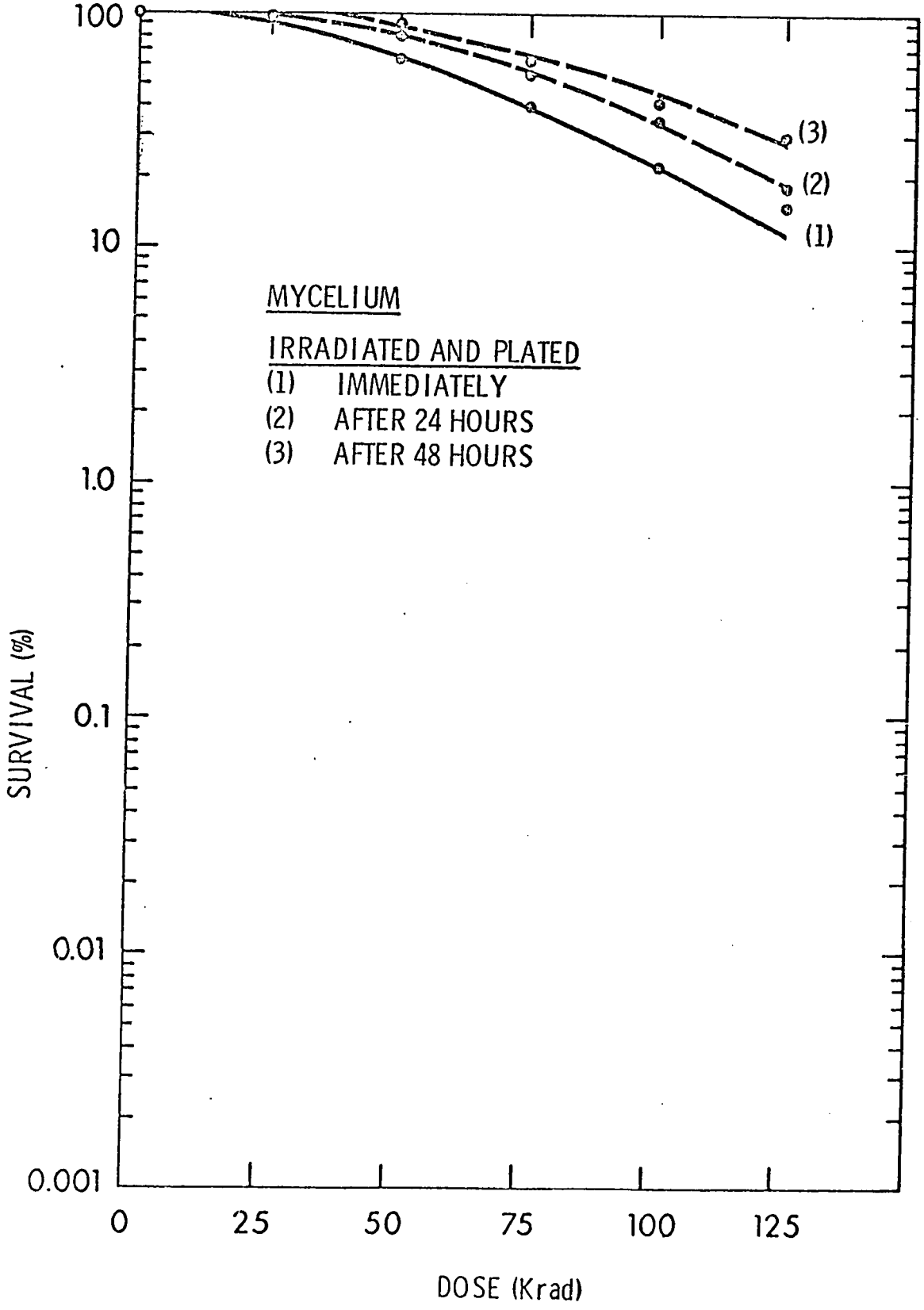

FIG.32. Survival of mycelium of A.flavus as influenced by two different intervals of time between irradiation and plating on Czapek agar medium.

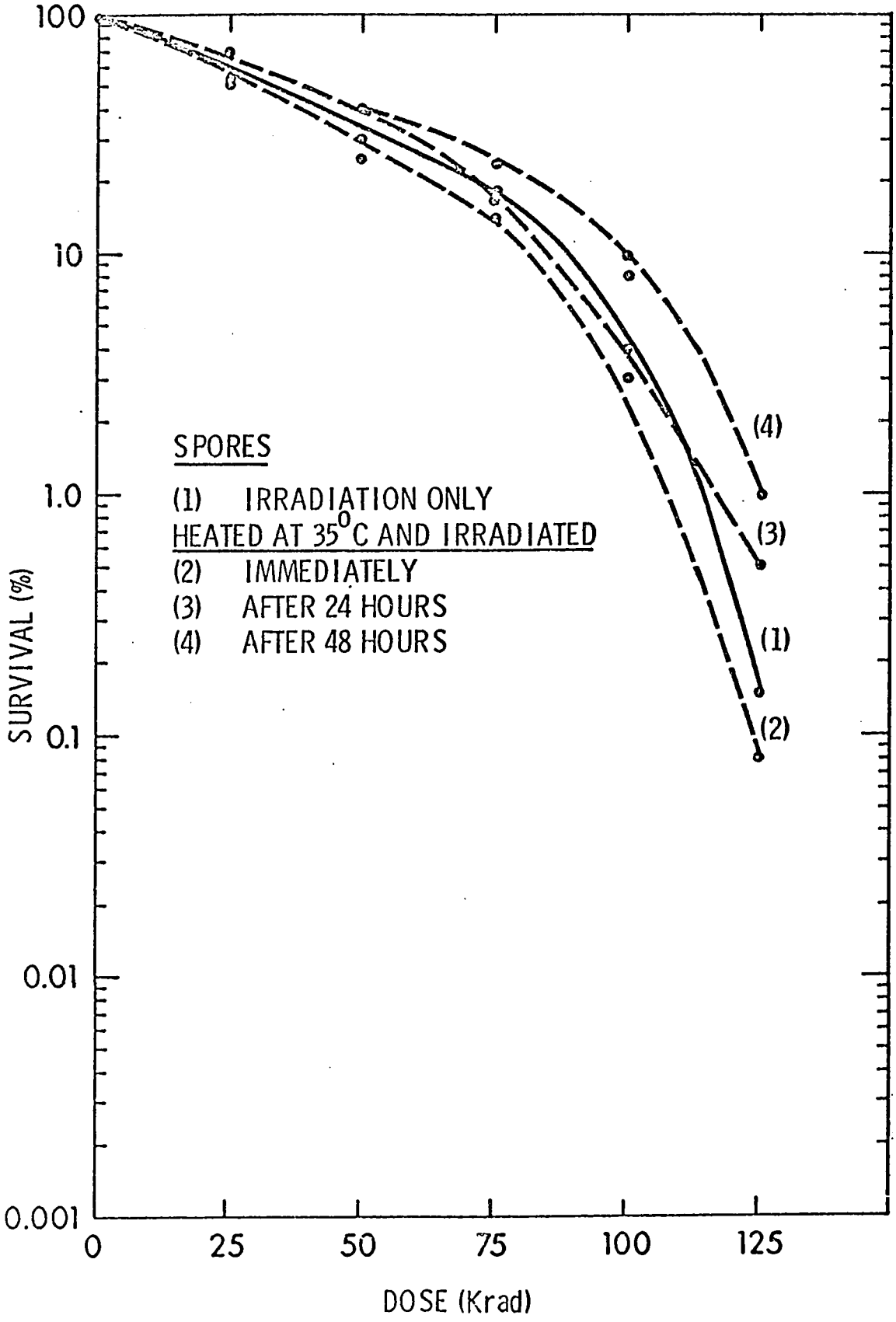

FIG.33. Survival of spores of A.flavus as influenced by two different intervals of time between heating at 35°C and irradiation at different doses.

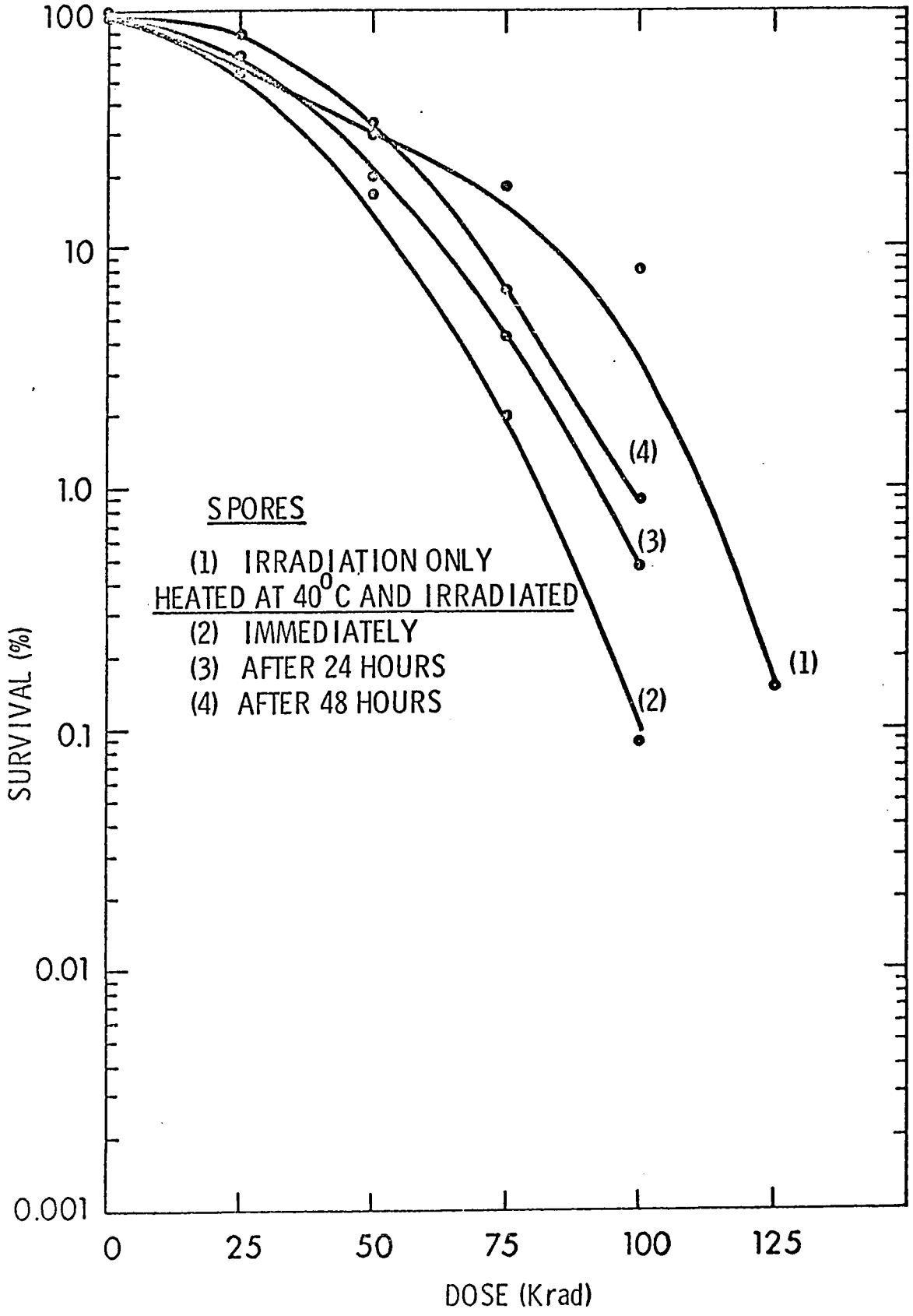

FIG.34. Survival of spores of A.flavus as influenced by two different intervals of time between heating at 40°C and irradiation at different doses.

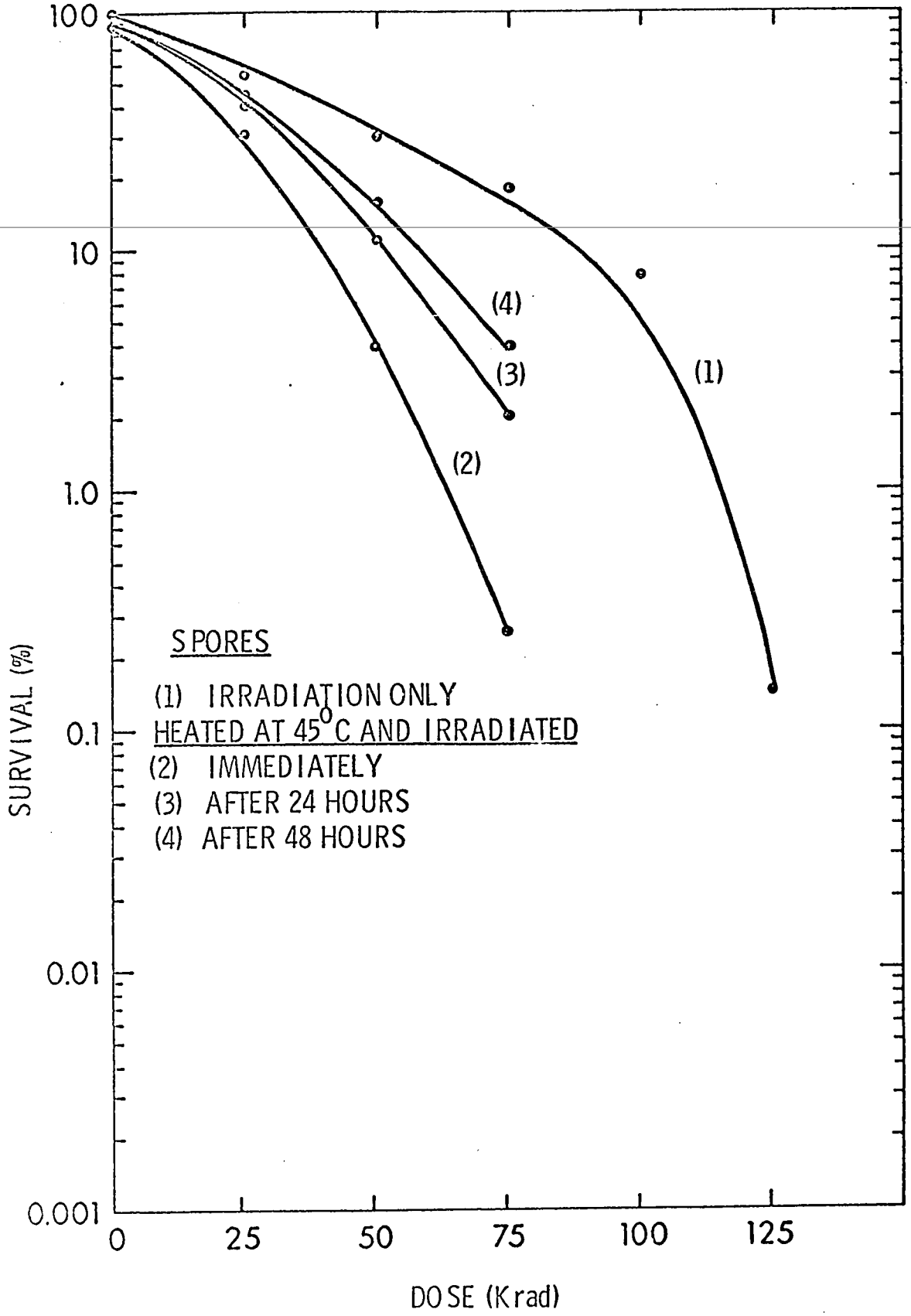

FIG.35. Survival of spores of A.flavus as influenced by two different intervals of time between heating at 45°C and irradiation at different doses.

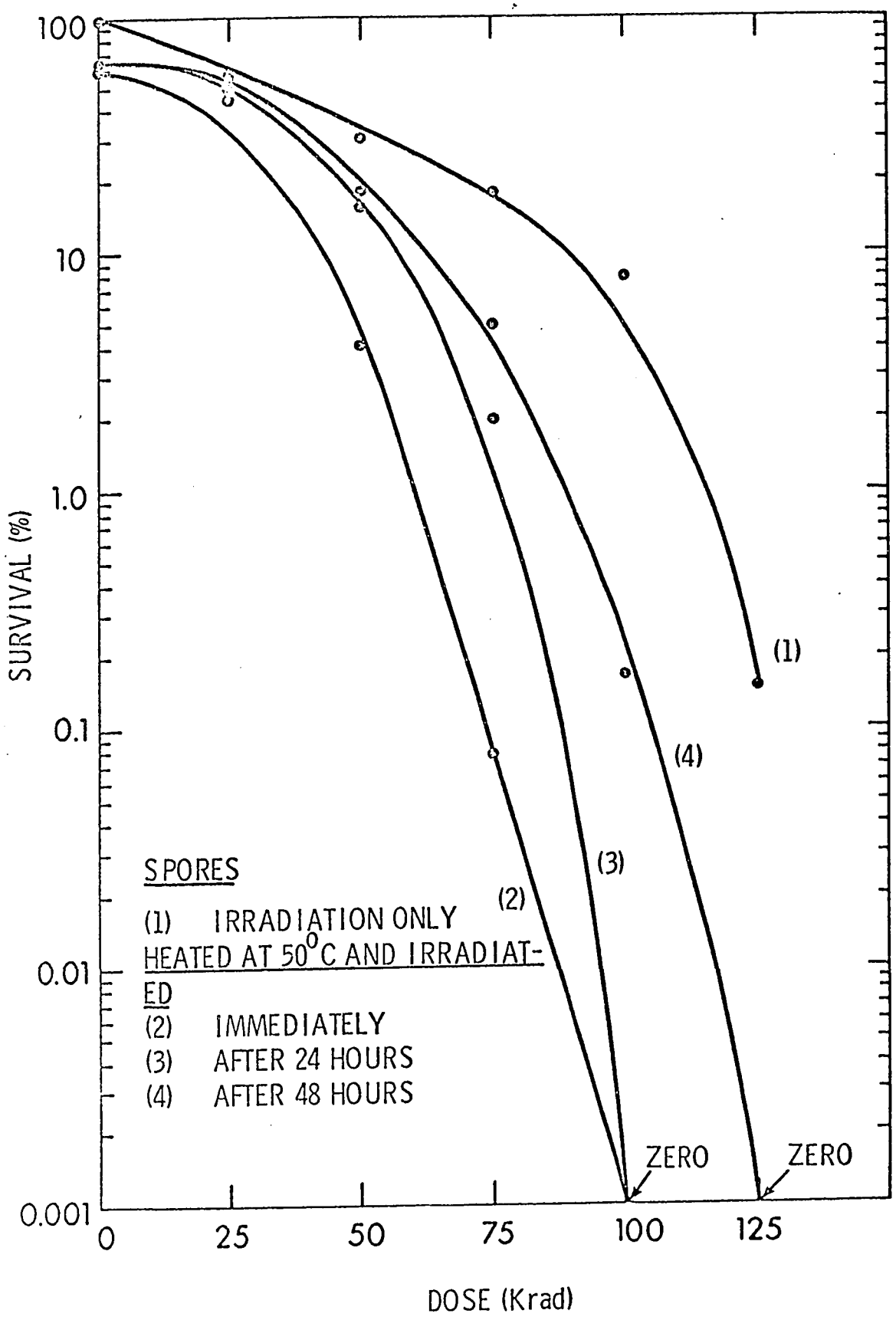

FIG.36. Survival of spores of A.flavus as influenced by two different intervals of time between heating at 50°C and irradiation at different doses.

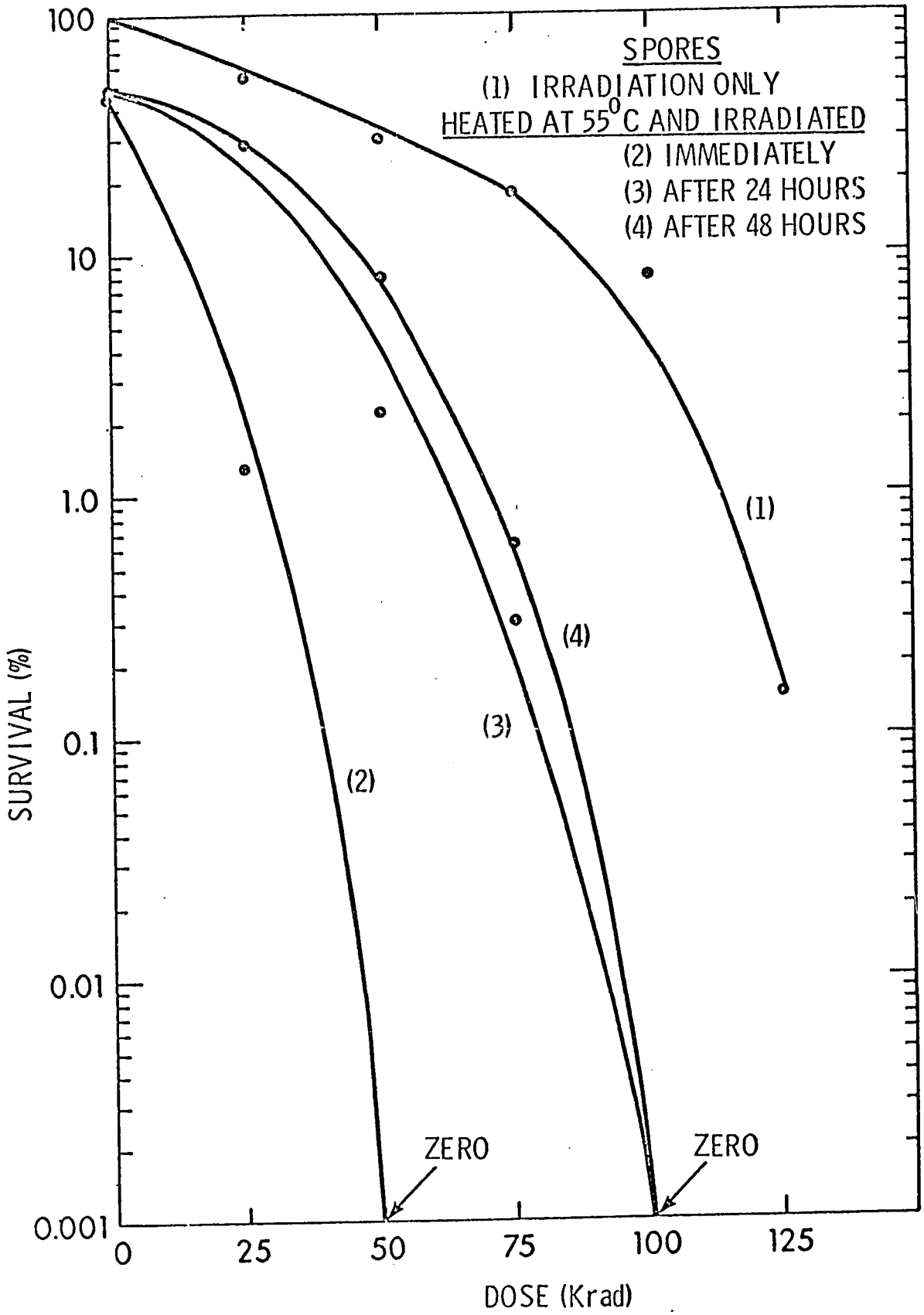

FIG. 37. Survival of spores of A. flavus as influenced by two different intervals of time between heating at 55°C and irradiation at different doses.

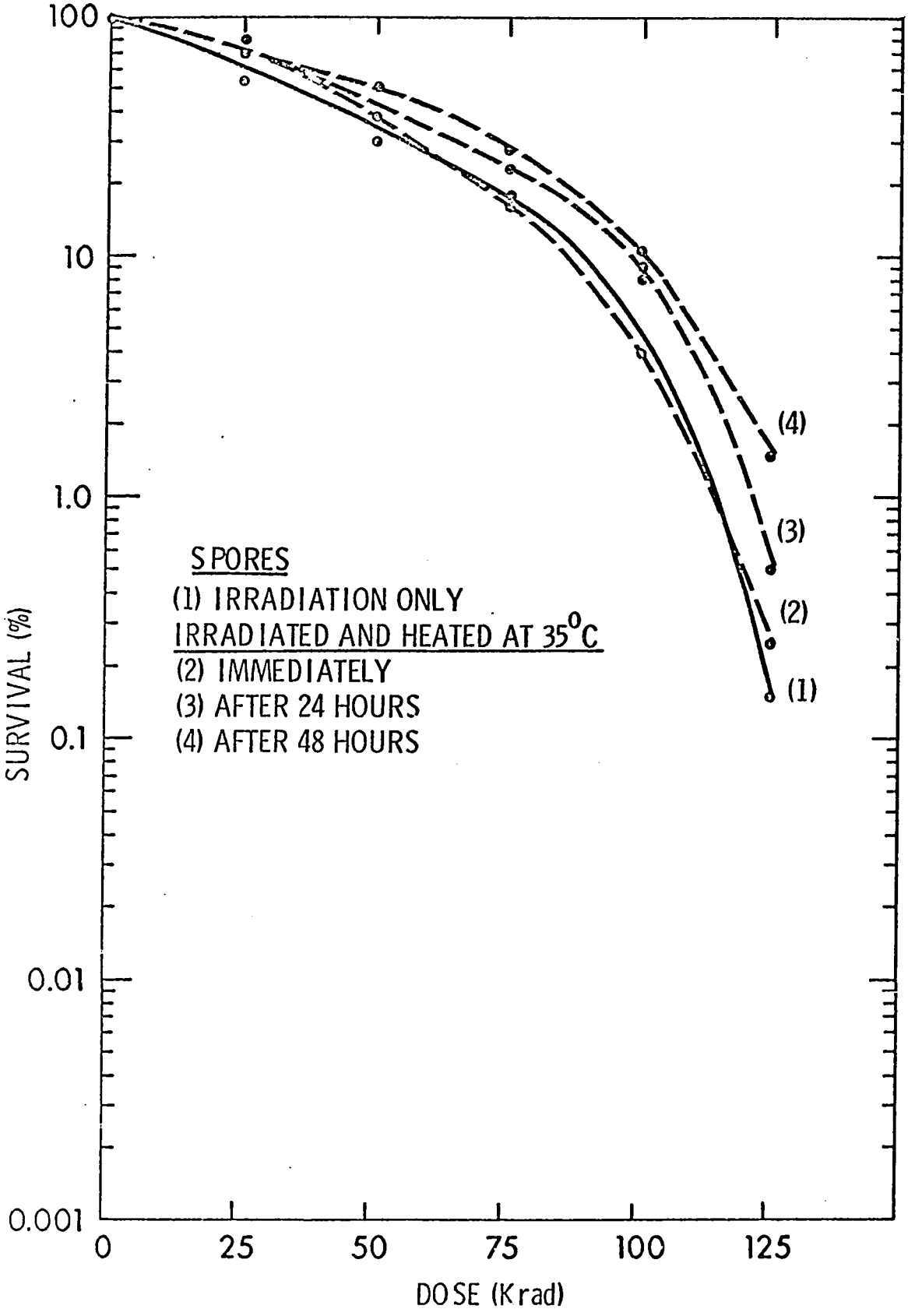

FIG. 38. Survival of spores of A. flavus as influenced by two different intervals of time between irradiation at different doses and heating at 35°C.

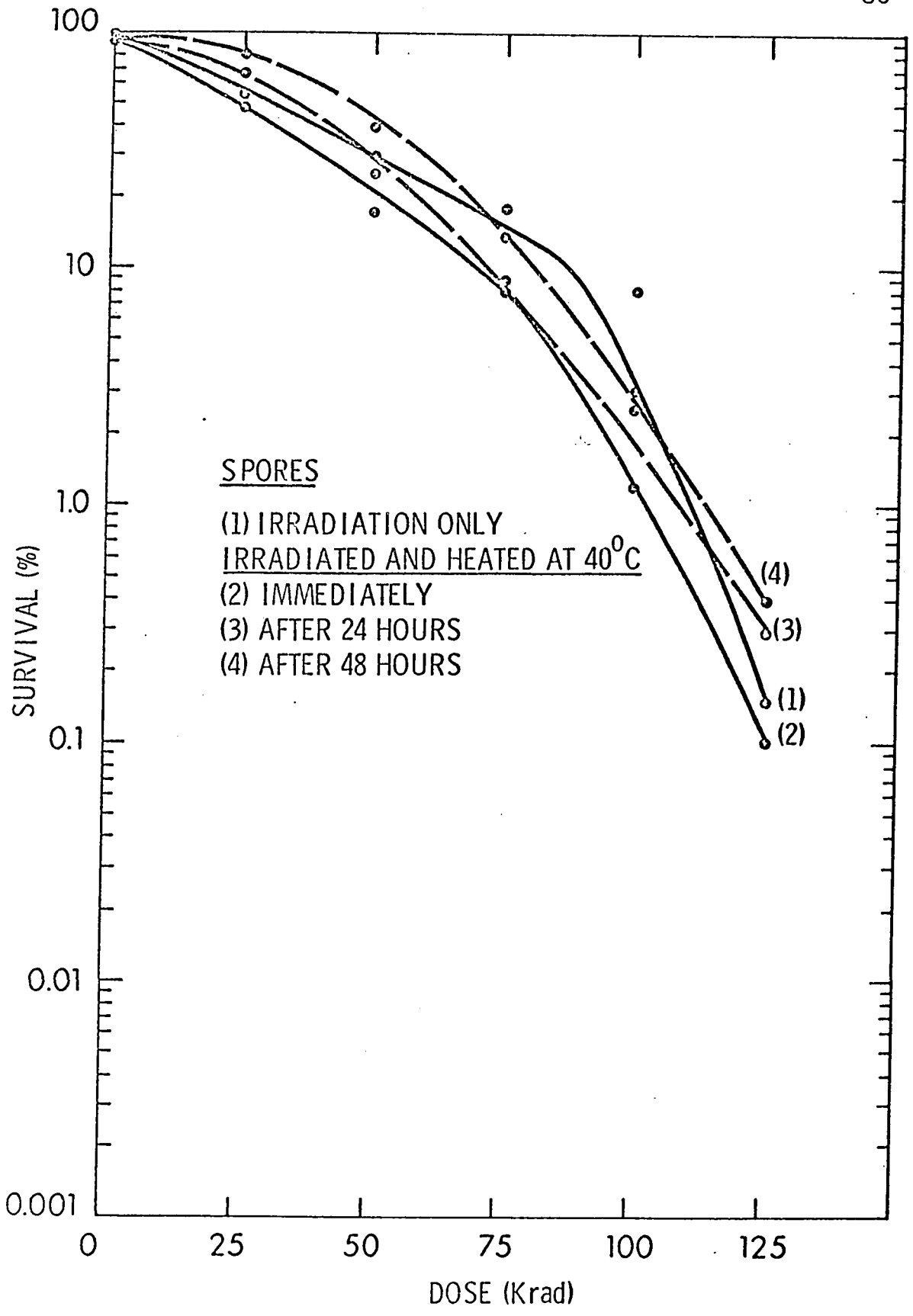

FIG. 39. Survival of spores of A. flavus as influenced by two different intervals of time between irradiation at different doses and heating at 40°C.

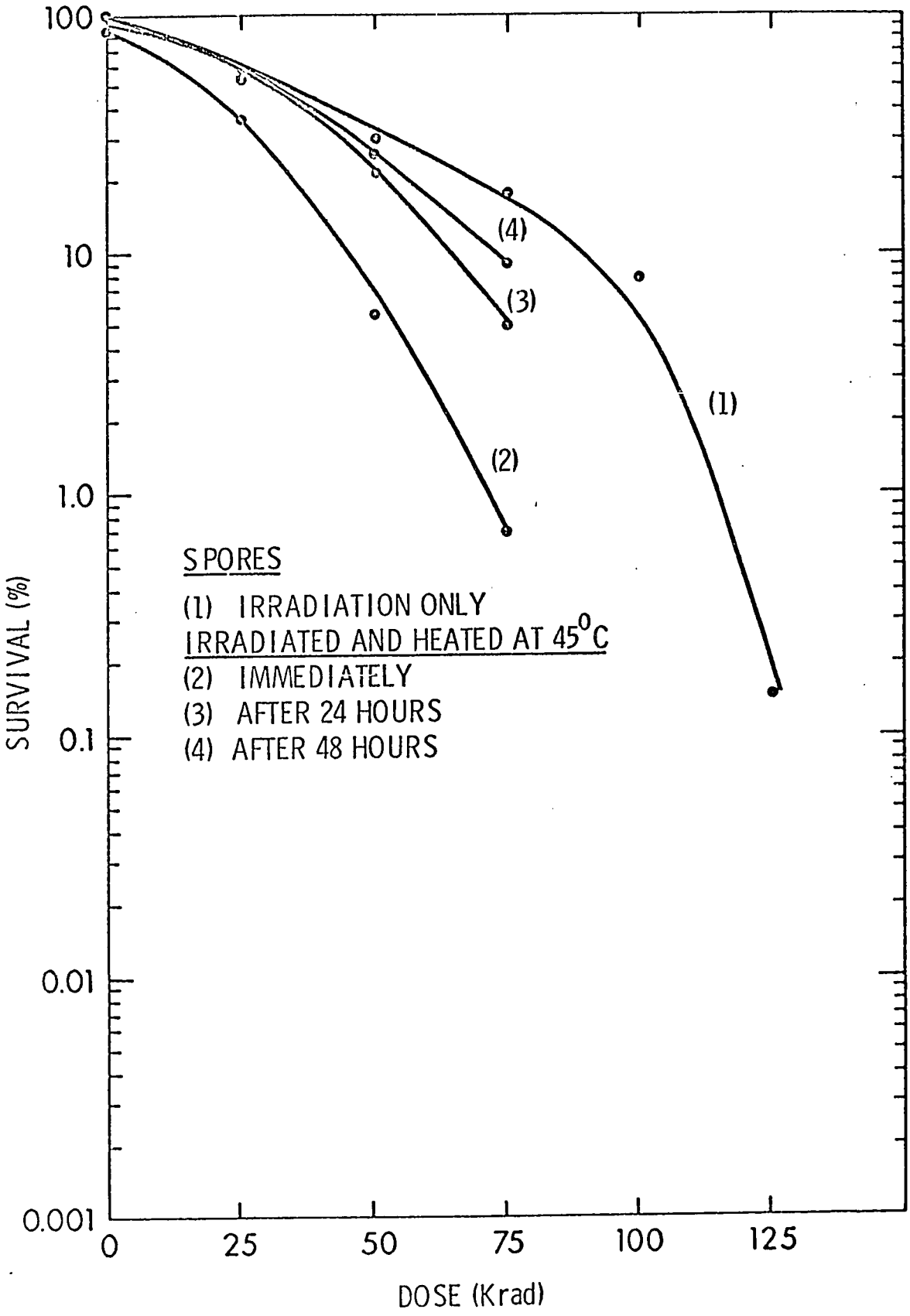

FIG.40. Survival of spores of A.flavus as influenced by two different intervals of time between irradiation at different doses and heating at 45°C.

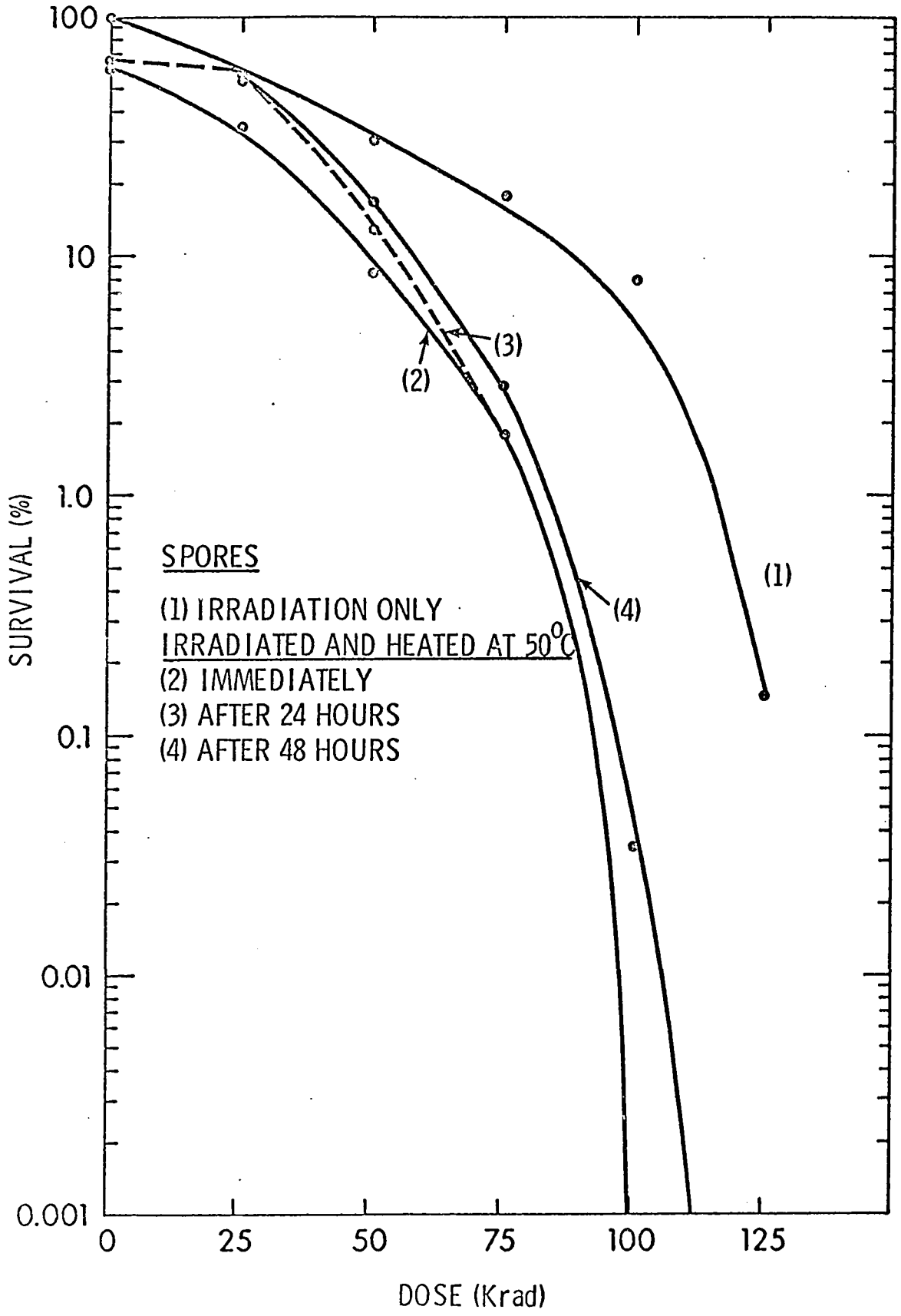

FIG.41. Survival of spores of A.flavus as influenced by two different intervals of time between irradiation at different doses and heating at 50°c.

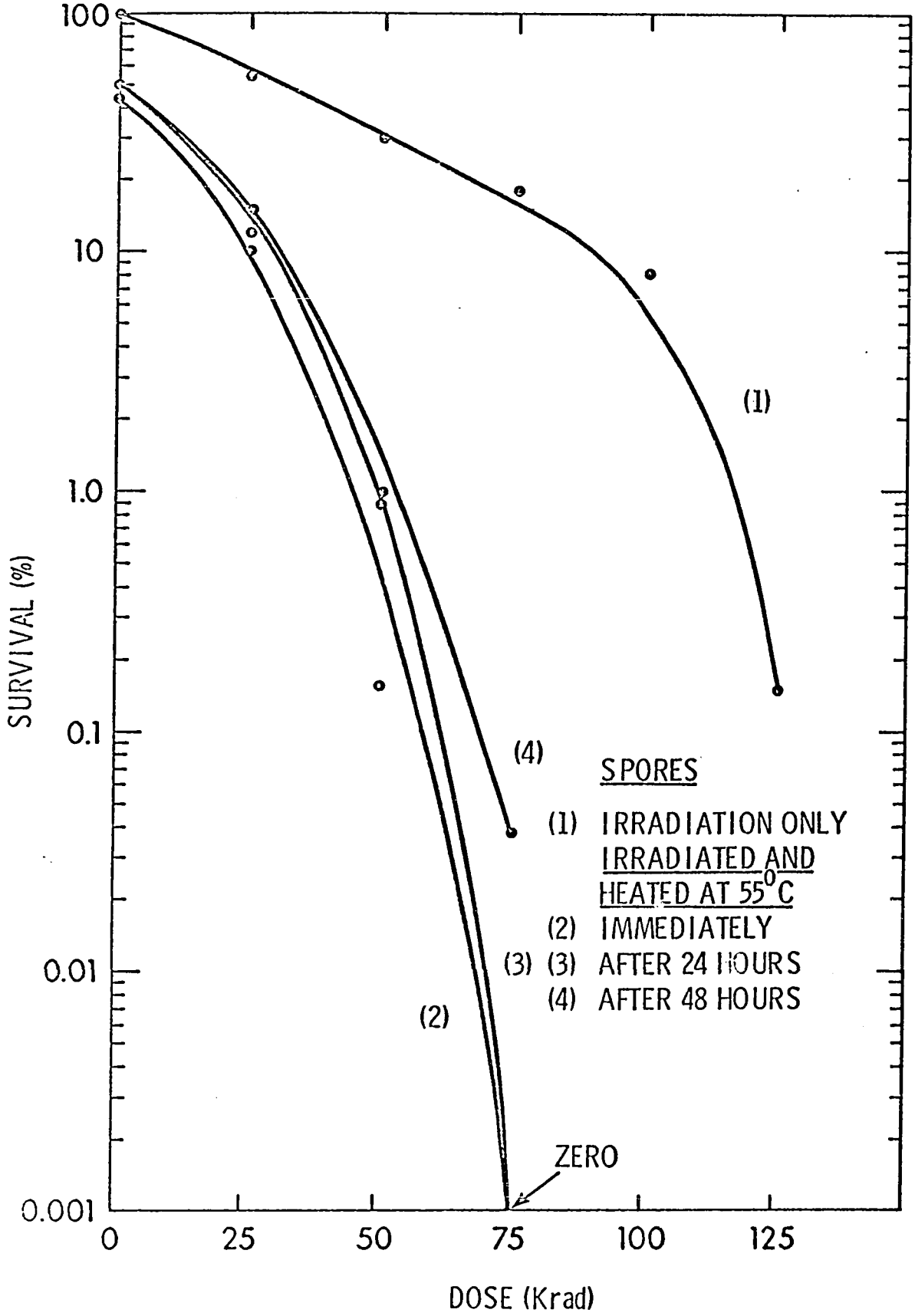

FIG.42. Survival of spores of A.flavus as influenced by two different intervals of time between irradiation at different doses and heating at 55°C.

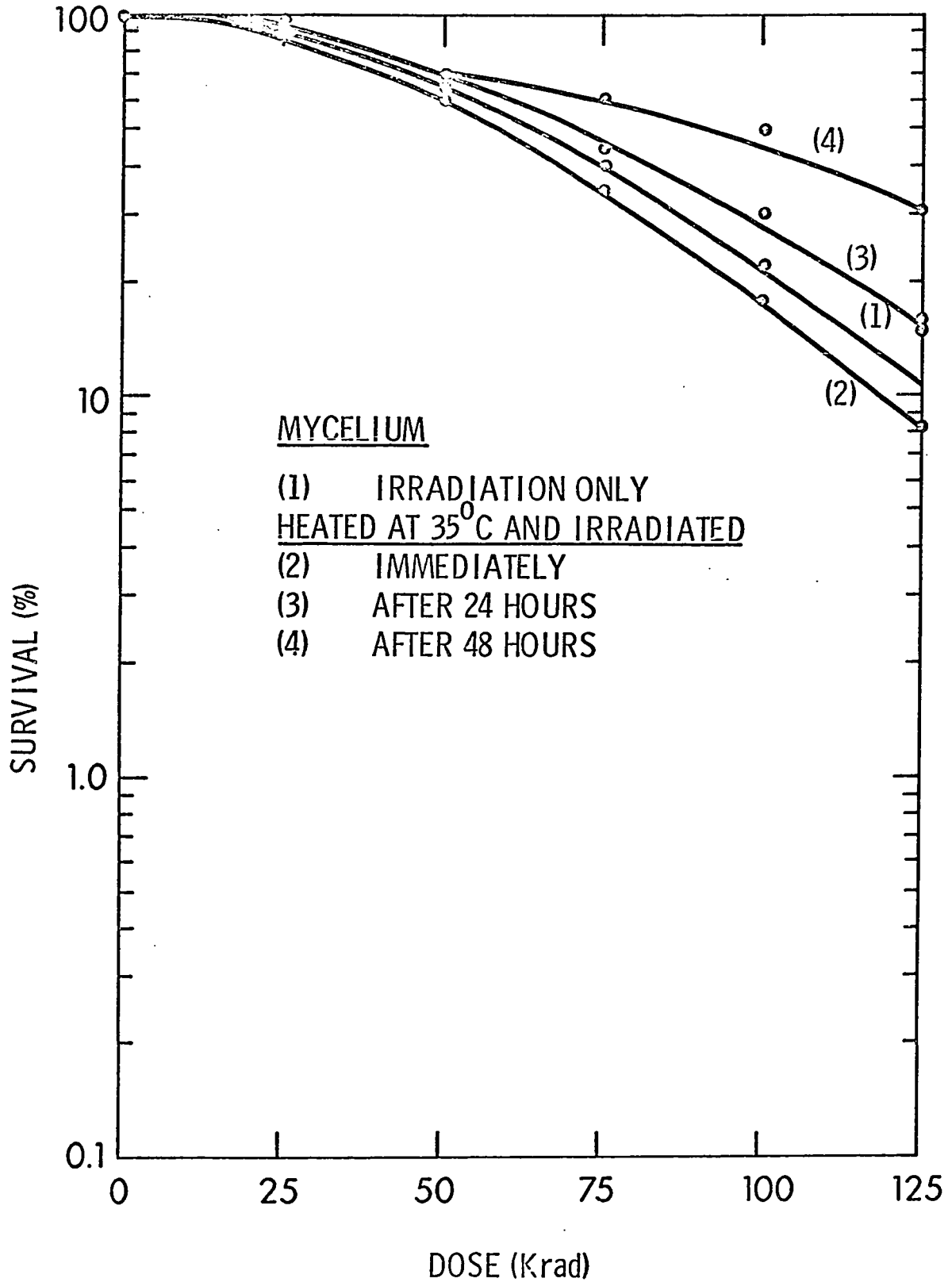

FIG.43. Survival of mycelium of A.flavus as influenced by two different intervals of time between heating at 35°C and irradiation at different doses.

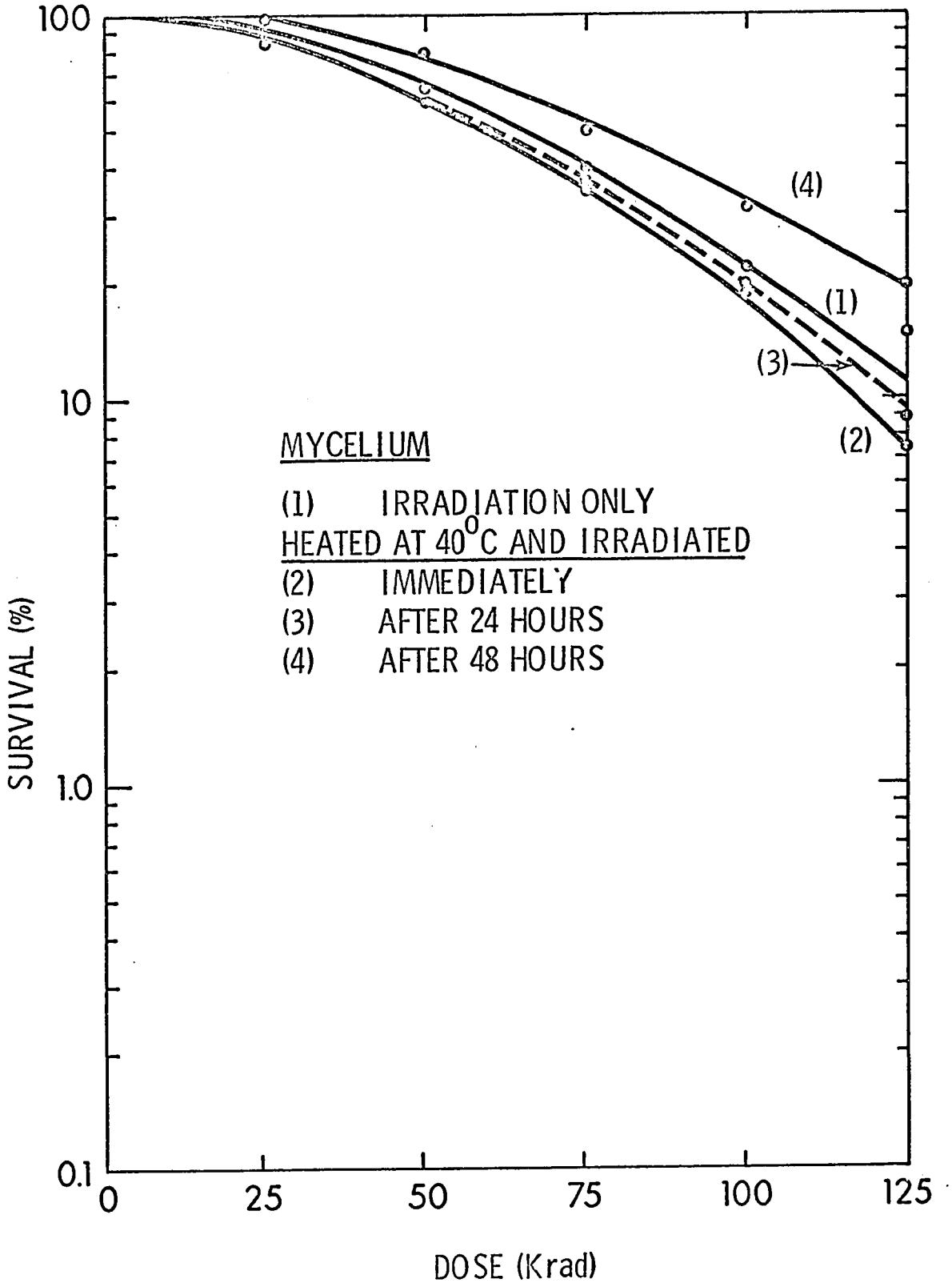

FIG.44. Survival of mycelium of A.flavus as influenced by two different intervals of time between heating at 40°C and irradiation at different doses.

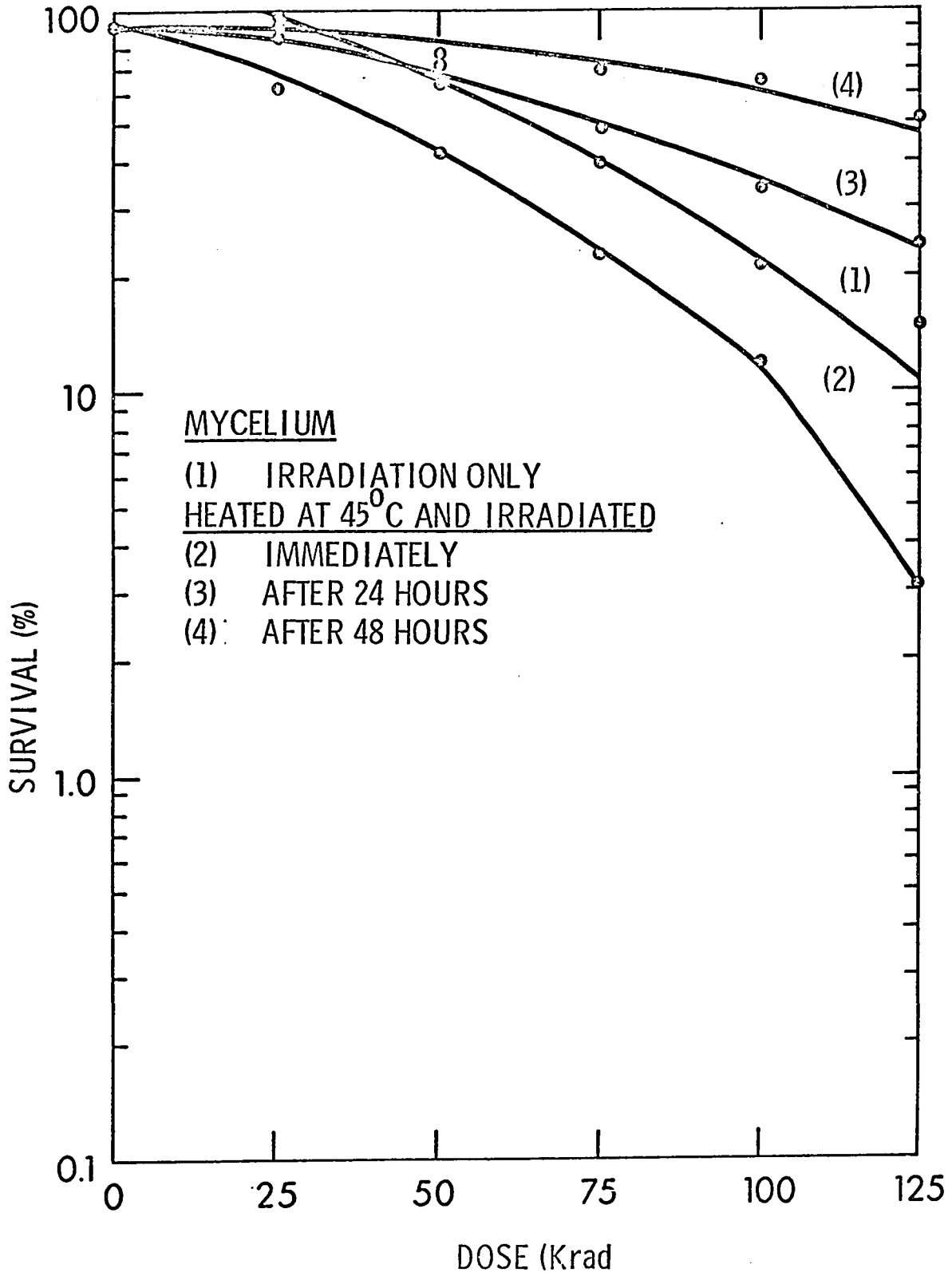

FIG. 45 Survival of mycelium of A. flavus as influenced by two different intervals of time between heating at 45°C and irradiation at different doses.

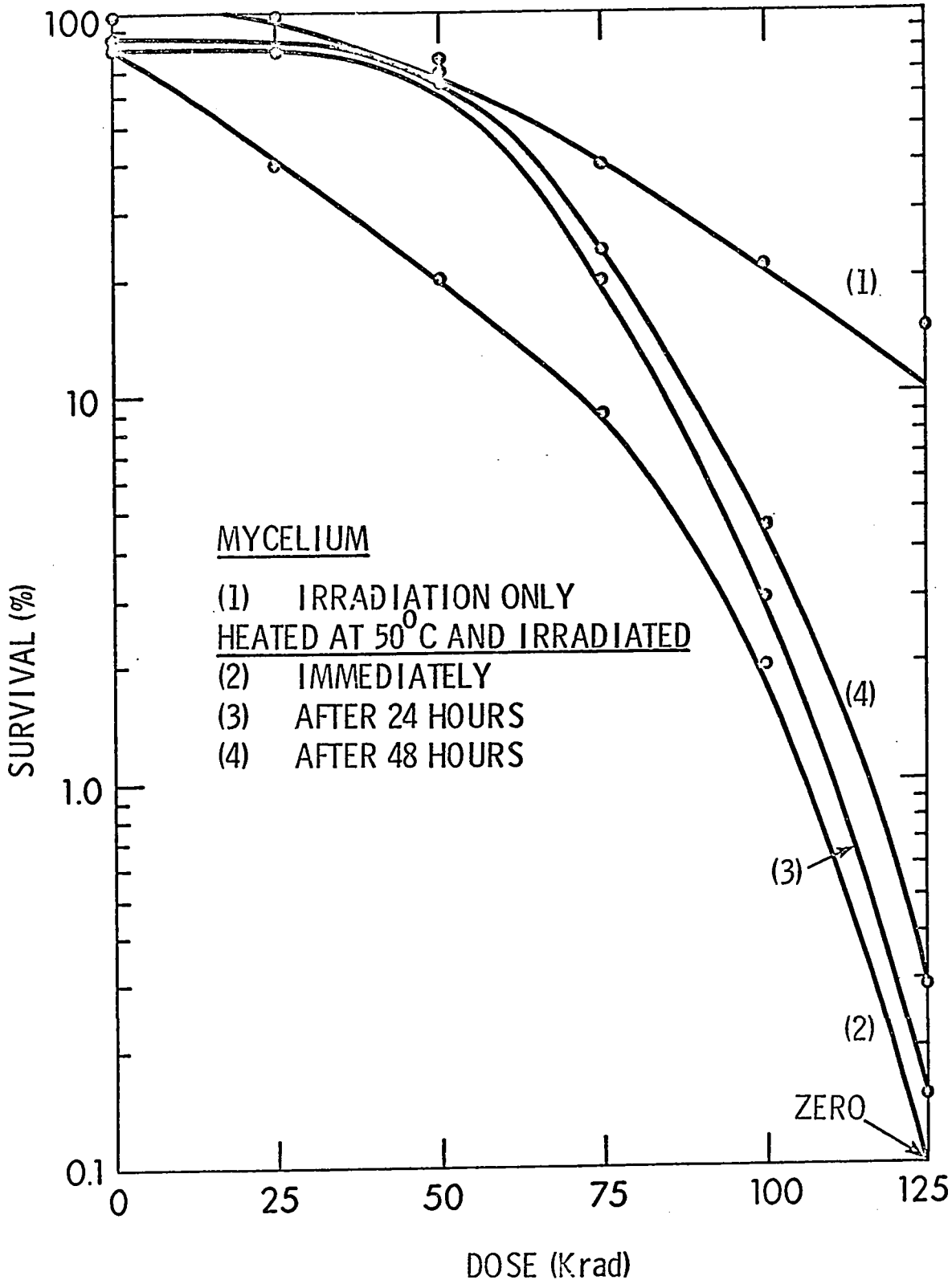

FIG.46 Survival of mycelium of A.flavus as influenced by two different intervals of time between heating at 50°C and irradiation at different doses.

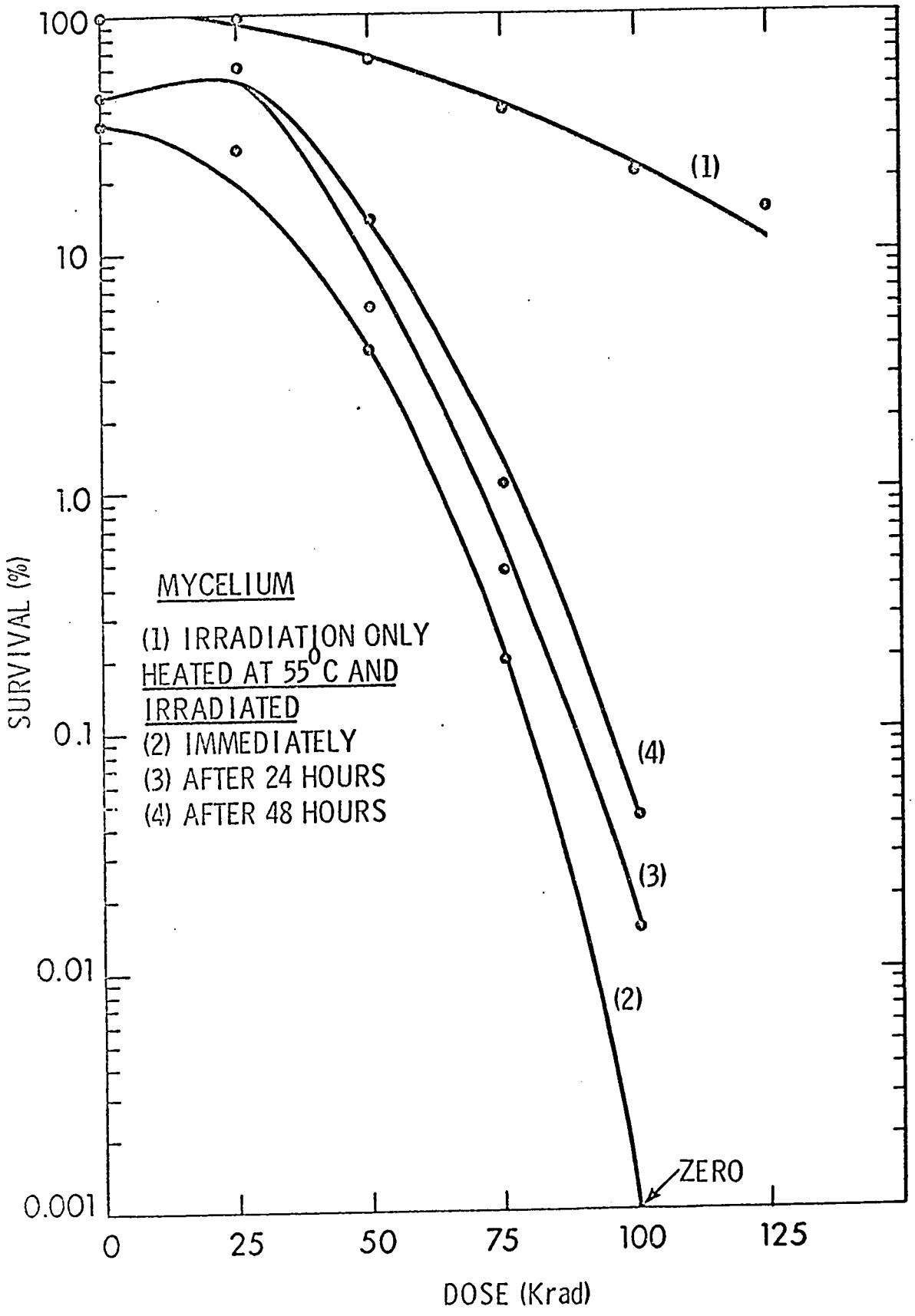

FIG.47. Survival of mycelium of A.flavus as influenced by two different intervals of time between heating at 55°C and irradiation at different doses.

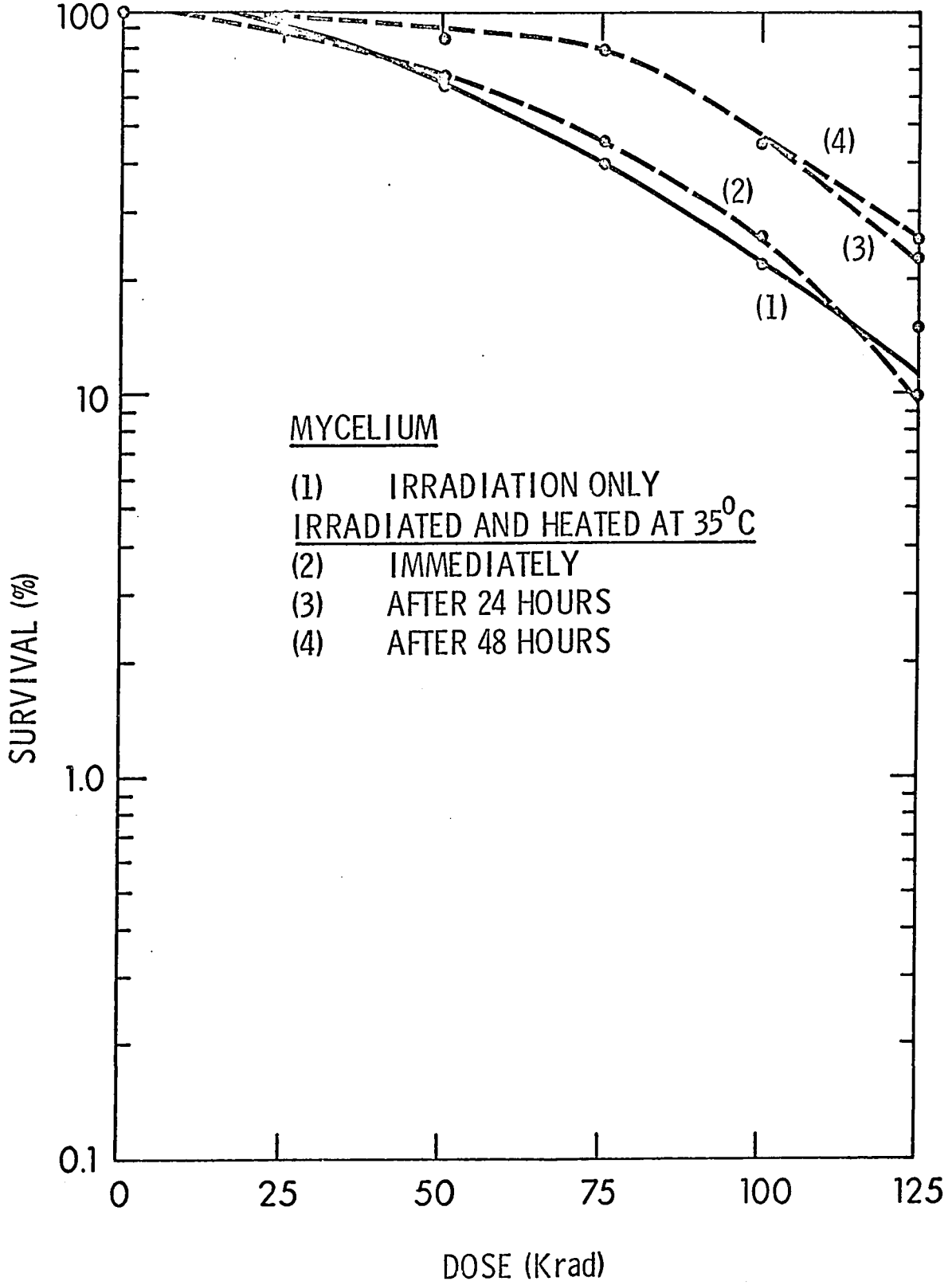

FIG.48. Survival of mycelium of A.flavus as influenced by two different intervals of time between irradiation at different doses and heating at 35°C.

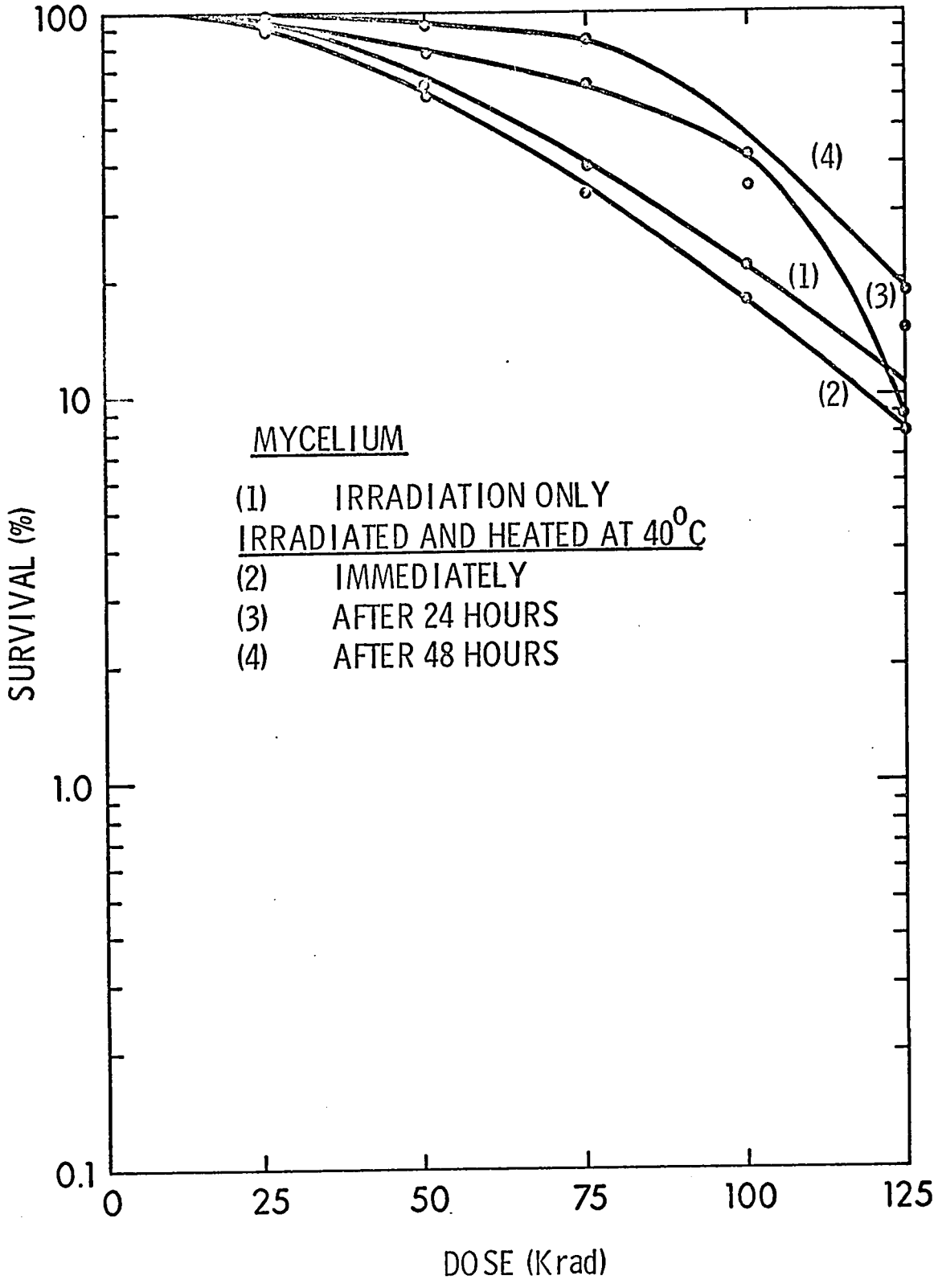

FIG.49. Survival of mycelium of A.flavus as influenced by two different intervals of time between irradiation at different doses and heating at 40°C.

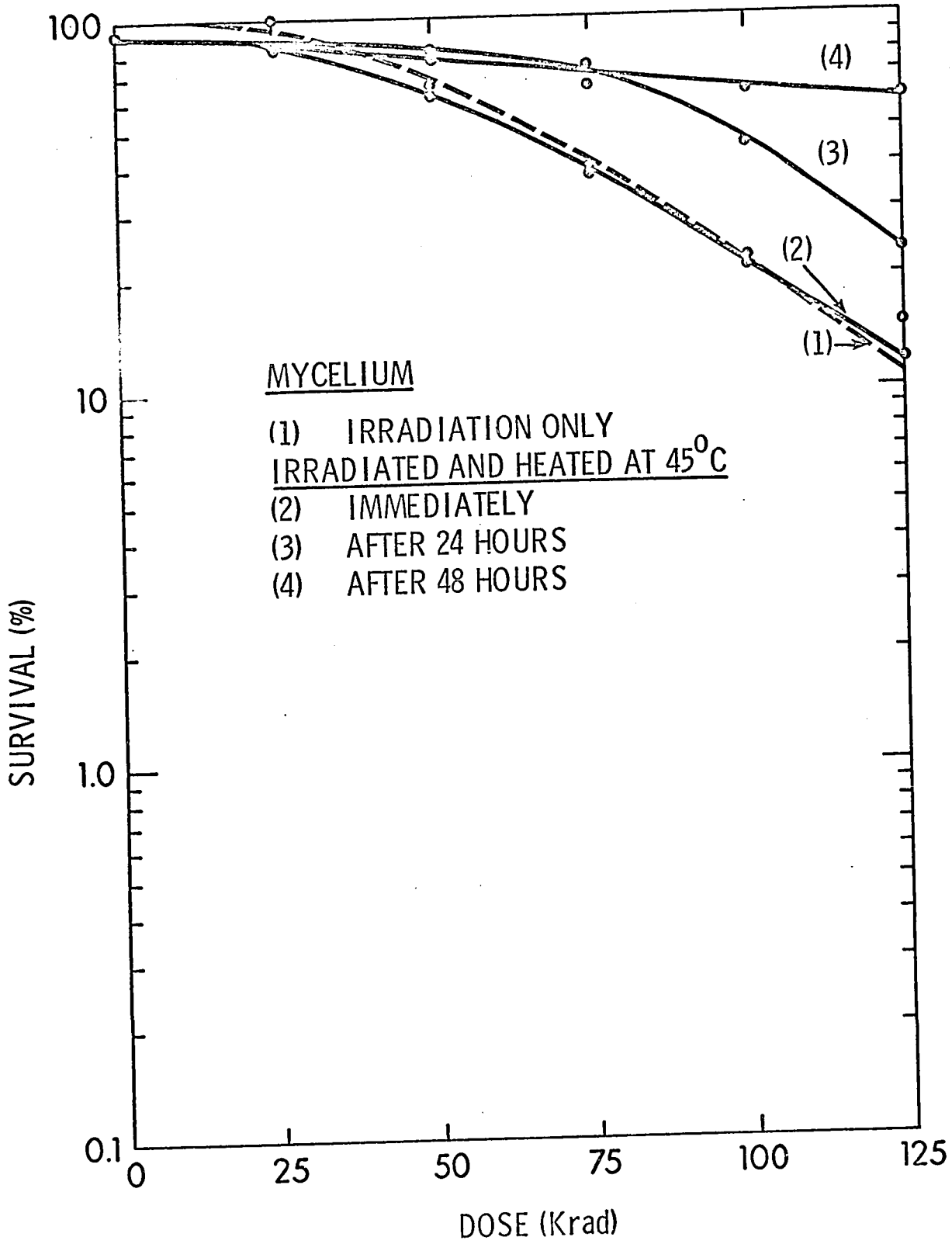

FIG. 50. Survival of mycelium of A. flavus as influenced by two different intervals of time between irradiation at different doses and heating at 45°C.

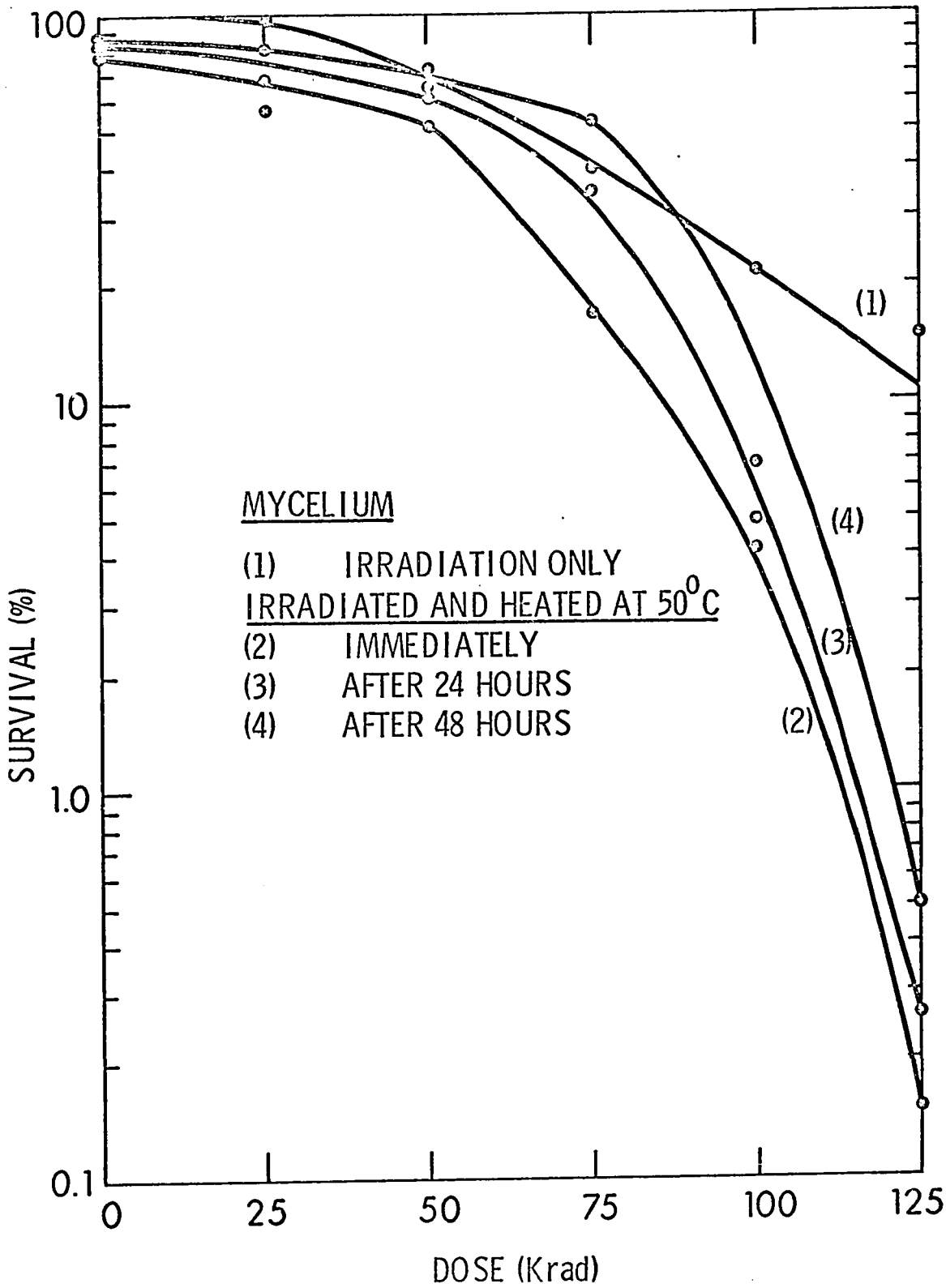

FIG. 51. Survival of mycelium of A. flavus as influenced by two different intervals of time between irradiation at different doses and heating at 50°C.

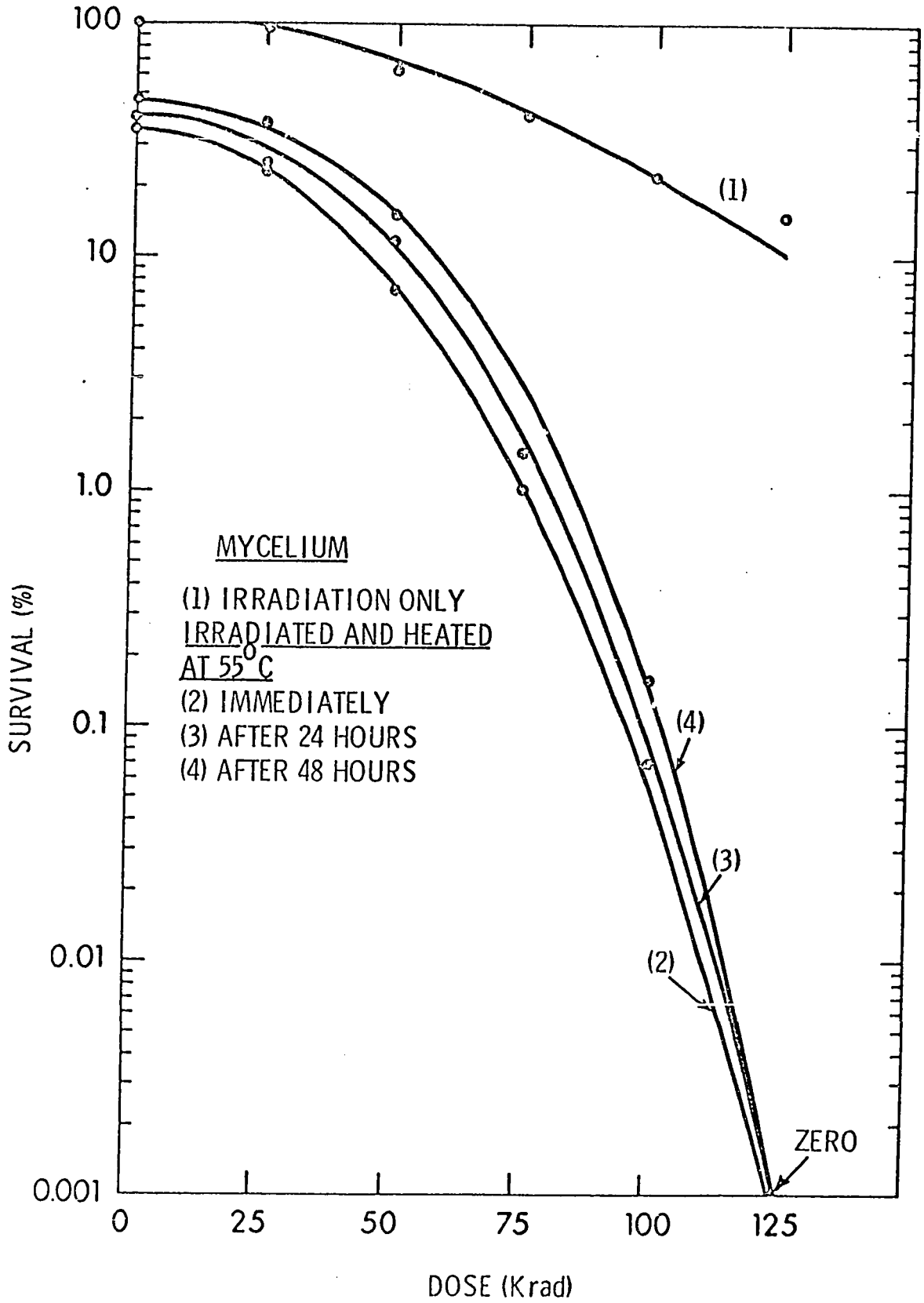

FIG. 52. Survival of mycelium of A. flavus as influenced by two different intervals of time between irradiation at different doses and heating at 55°C.

DISCUSSION

Spores of Penicilli and Aspergilli were selected for investigations concerning their in vitro sensitivities to gamma irradiation because they are easily obtained in large quantities, are readily dispersed in suspension, and are more homogeneous than other fungal structures with regard to irradiation resistance.

The irradiation survival curves followed a sigmoidal pattern, indicating that more than one event or "hit" was necessary to inactivate the colony-forming ability of a spore. An initial shoulder of resistance was followed by an exponential portion. The width of the shoulder was different for different species, indicating a variable degree of resistance in the spore populations. A sharp decline in the exponential portion of the survival curves for A.flavus and A.wentii indicates the existence of a threshold dose which must be applied to initiate inactivation. An analysis of survival curve parameters, i.e., extrapolation numbers and slopes, based on target theory or its modifications, does not appear likely to reveal the nature of the sensitive sites or targets within the spores of these fungi nor the mechanism of radiation damage.

The sigmoidal survival curves obtained for these spores are simply interpreted to indicate that damage leading to inactivation is cumulative, i.e., several events are necessary to bring about inactivation of a spore. How and where the damage is accumulated, cannot be ascertained from

these curves, but studies of other investigators (7, 8, 9, 48, 117) have implicated the nucleus as being the primary site of radiation damage in fungal spores. In the investigations cited, multinucleate spores yielded sigmoid survival curves, while uniculeate spores gave exponential survival curves. If these are taken as general findings, then it is to be assumed that spores in these species were multinucleate. Since no cytological investigations were carried out this assumption is not easy to rule out. However, spores (conidia) in species of Penicillium and Aspergillus are generally uninucleate and, therefore, it appears more likely that sigmoidal curves are a result of inactivation of several sensitive sites within one nucleus. Plating errors due to clumps would also result in sigmoid curves (105). However, in this study, the suspensions used for irradiation were free of spore clumps.

The relative contribution of indirect damage to the fungal spores, i.e., through the formation of free radicals of water, will be much less because of the low water content of the spores.

The irradiation doses required for zero survival of spores of Penicilli and Aspergilli are sufficiently high to be impractical for use on wheat grains because of the possibility of inducing nutritionally unwholesome changes. Thus, it was considered important that detailed investigations should be carried out on means of sensitizing these fungi to gamma irradiation. For this purpose Aspergillus flavus was

selected because of the following characteristics: it is very important in grain spoilage; it produces spores abundantly; it is not precise in nutritional requirements; the green colored spores facilitated counting, and its two-component survival curve had a sharply declining exponential portion and a well defined shoulder, so that any change in its resistance is readily reflected as a change in the shape of the survival curve.

Mycelium, as well as spores of A.flavus, has a reproductive function and, therefore, the prevention of grain spoilage can be achieved only by the destruction of both structures. Quantitative studies on the radiosensitivity of the mycelium in fungi are lacking, primarily, because of their multicellularity and the difficulties in quantitating them on a per cell basis. Thus, standardization of a method for irradiation of mycelium was important.

In order to select a suitable stage of mycelium for irradiation, the spores were incubated in liquid Czapek medium until various stages of germination were obtained. The spores which were irradiated after 6 hours of incubation were found to be more sensitive to irradiation when compared to spores which were not incubated. A microscopic examination revealed that at this stage there was no germination tube, but a swelling had occurred. The survival curve had a small shoulder while the exponential decline was prominent. The irradiation sensitivity at this stage of

germination has also been reported by Weijer (125) for Neurospora crassa and by Wilson (130) for conidia of Penicillium atrovenetum. Weijer reported that, in well aerated N.crassa conidial suspensions in a minimal medium, DNA replication took place within the first six hours of incubation at 30°C, and this DNA replication was found to be paralleled by an increase in radiosensitivity measured in terms of conidial kill. She also stated that fluctuation in the percentage of conidial kill appeared to be due to a fluctuation in radiosensitivity of the nucleus. The conidium did not produce a hyphal germination tube until after 7 hours of incubation, indicating that the target size of the conidium remained unchanged. The nucleus, according to her, underwent rapid changes with respect to shape and DNA content. Thus, she concluded that a rise in conidial radiosensitivity coincided with the duplication of DNA.

Wilson (130) reported that spores of P.atrovenetum, x-irradiated at various stages of germination, showed that x-ray sensitivity increased during early stages of germination, when spores were undergoing swelling. Maximum sensitivity of spores occurred about 5-6 hours after incubation, but he was not able to implicate any particular process as the basis of this x-ray sensitivity.

The resistance of spores of A.flavus to γ-irradiation began to increase after 6 hours of incubation (Fig. 11). After 24 hours of incubation there was a disappearance of an

exponential portion and the shoulder became more prominent. The spores, harvested after 24 hours, had produced a 2-celled germ tube and showed a tendency to aggregate. However, they could be easily separated by shaking in liquid. The increase in radiation resistance after 12 and 24 hours of incubation might be attributed to the increase in the cell number, or the occurrence of some radioprotective substances, or the increase in the number and size of targets due to increase in DNA, RNA and protein contents.

The inseparable aggregation of A.flavus spores, observed during the later stages of germination, is a phenomenon which commonly occurs when molds are grown in submerged shake cultures. It has been suggested (24) that germinated spores trap each other in an agitated medium, and form a nucleus around which the hyphae develop to form a pellet. This mechanical trapping becomes a conspicuous phenomenon at 36 hours in a shake culture. Aggregation of this type can lead to errors in the determination of survival if colony-forming ability is used as the criterion. This assay depends on the ability to distinguish each individual germinating spore. Aggregation can lead to underestimation of the number of germinating units in a suspension, since the plating of a clump of several germinating units results in only a single colony. It can also cause an overestimation of survival, after gamma irradiation or heat treatment, because the presence of just one viable cell in a clump is sufficient to produce a colony upon

plating, thereby masking any inactivation of spores in the clump. Based upon these considerations, spores incubated for 24 hours were selected for mycelial radiosensitization studies.

The selection of chemicals for radiosensitization of A.flavus was based primarily on the literature reports that these chemicals, when present during irradiation, can enhance the lethal effects of irradiation on bacteria and yeast. The chemicals selected include vitamin K_5 (a food preservative), iodoacetamide and iodoacetic acid (enzyme poisons), inorganic salts, i.e., NaCl, KCl, $CaCl_2$ with different halogen substitutions and some oxidizing agents, e.g., $NaNO_3$ and KNO_2. Iodized salt was included to determine whether the presence of iodine as an admixture has any relation to radiosensitization. Concentrations, in which these chemicals were used, were based on values quoted for bacteria. In addition,preliminary tests were carried out with various concentrations and the ones which were selected were either non-toxic or only slightly toxic to the spores of A.flavus, when exposed for a period which was required for the administration of the maximum dose of irradiation.

Radiosensitization of spores or mycelium involved the use of chemicals in combination with irradiation to produce a synergistic effect. An understanding of the mechanism through which irradiation damages these fungal structures is important to the understanding of the role of chemicals in enhancing the lethal effects. It is well known

that all components of a cell can be affected by irradiation. The death of the reproductive cell, caused by exposure to irradiation, is usually attributed to genetic damage, notably to DNA. Some experiments with bacteria have indicated that damage to the cell surface by x-irradiation might be involved (2, 51). Leakage of inorganic phosphate, ninhydrin-reactive material and substances absorbed at 260 mμ have been reported from cells of Saccharomyces cerevisiae, treated with ionizing radiation (70). The amount of inorganic phosphate released depended on the radiation dose, the temperature and pH during irradiation. The same workers observed that compounds which protected microorganisms and mammals against the lethal effect of ionizing radiation, also inhibited the radiation-induced release of inorganic phosphate from yeast. Other workers (100, 118, 119) reported that the permeability of yeast cells to potassium ions and other small molecules increased after doses of radiation similar to those that caused reproductive inactivation. Pollard and Weller (89) suggested that the initial lesion of ionizing radiation in E.coli is the rupture of the attachment of the DNA growing point to the cell membrane. This lesion is followed by increased leakiness of the cell membrane, by cessation of synthetic activities involving DNA, and by degradation of DNA.

Myers and Karazin (83) concluded that the loss of reproductive survival in irradiated yeast cells could not

be attributed to the kind of membrane damage that resulted in potassium leakage from the cells. Matsuyama et al (78), working on the combined action of NaCl and irradiation on E.coli, reported that some zones on the cell membrane were sensitive to this treatment. They postulated that chlorine radicals of NaCl play a significant role because such Na salts as sodium sulfate and sodium nitrate have no enhancing lethal effect. The increase in dose rate should result in higher concentration of the chlorine radicals because the concentration of OH radicals in aqueous solutions depends on dose rate. These Cl radicals may attack the inferred radiosensitive zones near the cell surface or the cell membrane. They also postulated that these radiosensitive sites may be different from the ordinary site within cells sensitive to toxic salt action. Namiki et al (86) reported that the Na or K ion is not responsible for the synergistic action of NaCl or KCl.

Sodium chloride, iodized salt, NaBr, NaI, KCl, KBr, KI, $CaBr_2$, CaI_2, $NaNO_2$, $NaNO_3$, iodoacetic acid, iodoacetamide and vitamin K_5 enhanced the lethal effects of γ-irradiation on the spores of A.flavus. Potassium nitrite did not show any synergistic effect, while $CaCl_2$ and $Ca(NO_3)_2$ afforded protection to spores against radiation damage. Since these compounds exerted their effect only when they were present during irradiation, it appears that their mode of action is mediated through the production of free radicals. Free radicals are produced as a result of radiolysis of water

molecules. These radicals possess a single unpaired orbital electron and are, therefore, very reactive, although extremely short-lived. The free radicals can react with other molecules and produce various other types of radicals through energy transfer reactions. For example, free radicals in NaCl are produced in the following reaction:

$$H_2O \longrightarrow H^{\circ} + OH^{\circ}$$

$$NaCl \longrightarrow Na^{+} + Cl^{-}$$

$$Cl^{-} + OH^{\circ} \longrightarrow Cl^{\circ} + OH^{-}$$

Behaviour of iodine compounds was noteworthy because they were invariably good radiosensitizers of spores of A.flavus. Mullenger et al (82) reported that, during the radiolysis of aqueous solutions of I^{-}, the following reactions occurred:

$$I^{-} + OH^{\circ} \longrightarrow I^{\circ} + OH^{-}$$

$$I^{\circ} + e^{-}_{aq} \longrightarrow I^{-}$$

$$I^{\circ} + I^{-} \longrightarrow I_2^{-}$$

$$2\, I_2^{-} \rightleftharpoons I^{-} + I_3^{-}$$

$$2\, I^{\circ} \rightleftharpoons I_2$$

They concluded that it is the short-lived I° free radical which led to sensitization. The other species, i.e., I^{-}, I_2, and I_3^{-}, are not involved because they are stable and would sensitize equally well whether present during or after irradiation.

Lee et al (69) reported that iodoacetic acid was a good sensitizer of Micrococcus radiodurans, which is highly radiation resistant. They concluded that radiolytic products

of iodoacetic acid, the iodide radicals, iodine atoms, the alkyl radicals and ions, may play a significant role in sensitization, owing to their high reactivities. The sensitizing effect of this compound appeared to depend more on its highly reactive free radicals than on its sulfhydryl-combining action alone.

A similar type of mechanism can be speculated with the spores of A.flavus. The question, however, remains that if only iodine radicals in case of compounds containing iodide molecule, and other halogen free radicals, in case of other compounds, are responsible for sensitization, then why are these compounds not equal in their sensitization capacities? It shall have to be assumed, therefore, that many characteristics of a particular compound may contribute towards its ability to radiosensitize. For example, in iodoacetamide, a highly polarized carbonyl group is also present in addition to halogen atom. The presence of this carbonyl group might be responsible for the higher radio-sensitizing capacity of this compound.

In simple inorganic salts, e.g., NaCl or KCl, it is possible that the contribution of free halogen radicals is more important, but at the same time the role of cations should also be considered. Potassium bromide and KI were equal in their sensitization capacities, while NaBr and NaI were not. No explanation can be offered for these differences.

The compounds with a bivalent cation Ca, behaved as

radioprotectors for spores of A.flavus at 25 krad. At higher doses, only $CaCl_2$ and $Ca(NO_3)_2$ gave protection, while $CaBr_2$ and CaI_2 behaved as radiosensitizers. Weijer (127) reported the protective effects of Ca in calcium gluconate and $CaCl_2$ on conidia of Neurospora. The radioprotective action of these compounds against the radiation death of the spores might be explained on the basis of the ability of Ca to form an autocomplex coacervate which, in turn, condenses to the plasma membrane. The synergistic action of $CaBr_2$ and CaI_2, at higher doses, might be attributed to free radicals of iodine, which probably reach the reaction site on the cell membrane before the Ca ions can form the complex. It appears that in these chemicals there is a combination of protective cations and synergistic anions.

It is not possible to explain the differences observed in the radiosensitization capacities of $NaNO_3$ and $NaNO_2$. The ability of these compounds to capture the solvated electrons (e^-_{aq}), which are produced as a result of radiolysis of water, might be held responsible for their radiosensitization (82). In vitamin K_5 the naphthol moiety is probably responsible for the sensitization by this compound.

The site of action of free radicals appears to be the protoplasmic membrane. It is unlikely that these compounds have penetrated deeply enough into the spores because of the short period of exposure. Spores are relatively inactive and the entry of these chemicals depends upon slow diffusion of cations and anions rather than through an active transport.

This phenomenon is also supported by the fact that no swelling of the spores was observable during this period. As discussed earlier, irradiation is known to increase the permeability of the cell membrane. If irradiation is carried out in the presence of chemicals, then it is likely that reactive free radicals are also contributing towards an increase in permeability, thus enhancing the extent of damage. This change in permeability may not be the cause for the reproductive death of the spores, as pointed out by Myers and Karazin (83) in irradiated yeast cells. It would appear, therefore, that a combination of extensive membrane damage and genetic damage resulted in an early death of spores. However, it is not possible to ascertain whether the site of damage on the cell surface by irradiation alone is the same as the site of the combined action of irradiation and chemicals.

The ability of these compounds to radiosensitize the spores of A.flavus differed from their sensitizing effect on mycelium. Not all the compounds, which sensitized the spores to gamma irradiation, were able to sensitize the mycelium. For example, NaBr did not show any sensitizing effect on mycelium. There is no explanation available for this difference. The two more striking differences between spores and mycelium were the extent of toxicity of these compounds, and their relative abilities to sensitize the mycelium and the spores.

Almost all the compounds exhibited toxicity to the

mycelium. The toxic action of hypertonic solutions of simple salts can be explained on the basis that the mycelial cells are in a state of very active metabolism and active ion transport. The high concentration of salts outside the cells causes toxicity through plasmolysis. Iodoacetic acid and iodoacetamide are toxic probably because of their ability to poison the -SH enzymes. The toxicity of these compounds obscures a true evaluation of the radiosensitizing capacity of these chemicals. But one fact is clear, that the combined effects of irradiation and of these chemicals on the mycelium are not additive but are synergistic. This rules out the possibility that toxicity alone contributes towards the observed sensitization. Some chemicals, which sensitized the spores, sensitized mycelium to a less degree.

Several reasons may be advanced for mycelium being less receptive to sensitization than spores, when subjected to the same treatments. Mycelium, being multicelled and multinucleate, has more targets to be inactivated. It may possess a more efficient repair mechanism or there may be some intracellular protective substances which contribute towards the lessening of the sensitization effect.

The mechanism of action of these chemicals in conjunction with irradiation appears to be the same on mycelium as on spores, that is, through the production of free radicals. However, in mycelium the free radicals in addition to damaging the cell membrane, might also contribute

towards the damage of the intracellular components, because it is likely that these chemicals can penetrate deep inside the mycelial cells during exposure to irradiation.

In summary, radiosensitization of the spores and mycelium of A.flavus can be achieved with various chemicals. The degree of sensitization appears to depend upon the nature of each chemical and on the radiation dose applied. Halogens are important in radiosensitization of the spores and mycelium. The physiological state of the structure to be sensitized also is very important in achieving a desired sensitizing effect. The free radicals appear to be the main contributors to radiation damage.

The spores and mycelia of A.flavus were considered heat-resistant and irradiation-sensitive when their survival values after each treatment were compared. Therefore, it was considered important to investigate the responses of these fungal structures to the combined effects of heat and gamma irradiation, applied simultaneously or in different sequences.

Thesynergistic effects of heat and gamma irradiation increased with an increase in temperature and irradiation dose. This increase was more pronounced at temperatures above 45°C. There was a difference in synergism depending upon the sequence of treatments. The preferential sequence of heat-irradiation inactivation also differed for spores and mycelium. With spores an application of heat at various temperatures, immediately followed by irradiation, was more

synergistic than the other two combinations. There were some unexplained discrepancies at lower temperatures (35° and 40°C), but at higher temperatures (45°, 50° and 55°C) application of heat before irradiation proved to be the most effective combination in inactivating the spores. The less effective combination was a simultaneous application of two treatments and the least effective was heating after irradiation. The survival curves of spores, exposed to lower temperatures and lower irradiation doses, showed an additive effect of these treatments, while at higher doses the treatments were synergistic in interaction.

With mycelium, a simultaneous application of heat and irradiation was the most effective lethal treatment, followed by combinations involving heating of spores before irradiation, and the least effective combination being the application of heat after irradiation. In this respect these results agree with those reported for yeast cells (116).

There are basic differences in the biophysical action of heat and irradiation on essential cell molecules. Radiation possesses extremely energetic quanta, which cause random ionizations and excitations in cell molecules, as well as in the surrounding medium, especially water. The latter causes the indirect effects of radiation which involve chemical oxidations and reductions. Heat is a relatively weak force affecting molecular organization, and does not disturb the atomic structure of the matter. Heat

disrupts weak hydrogen bonds and occasionally breaks stronger covalent bonds by intense Brownian movement of water molecules in the medium. It also puts stress on the biological molecules themselves by intensifying their rotational and vibrational behaviour.

Various theories have been advanced to explain the increased radiosensitivity at high temperatures. These have included such ideas as: a) increased diffusion of free radicals, b) increased target size due to thermal expansion, c) excitation of the biologically important molecules, d) formation of new radical species at high temperatures and, e) instability of molecular bonds at high temperatures.

Some of these factors may play a minor role in contributing to the overall effect. However, the model proposed by Adams and Pollard (1) seems to be widely accepted According to them, the denaturation of proteins by heat requires the rupturing of three adjacent residue bonds in the molecule. This causes the main chain to drift apart, and the molecule loses its biological configuration. If one or more of these bonds are broken by ionizing energy, the requirements for thermal energy are reduced.

In view of these facts, it is difficult to explain the different responses of spores and mycelia, with regard to the preferential sequence of various heat-irradiation combinations. It appears probable that spores, being dormant structures, are activated by a preliminary heat shock

to some degree and, as a result of this shock, they become more susceptible to damage by irradiation. This speculation is strengthened by observations that, when irradiation is applied 24 hours after heating, the spores become more resistant to inactivation by irradiation. In mycleium, however, an efficient repair process appears to occur so that, if it is heated first and then irradiated, the repair processes will become operative. In the simultaneous application of heat and irradiation there is no time for the repair processes to become functional.

The spores and mycelia of A.flavus appeared to be able to repair the damage caused by sublethal doses of gamma irradiation, if they were held for some time under conditions that were not conducive to germination or growth. The extent of recovery from damage seemed to depend upon the period of time during which they were kept in distilled, demineralized water. The damage at lower doses (25 or 50 krad) was repaired within 24 hours, while at higher doses this repair process continued up to 48 hours.

Thus, if the heat and irradiation treatments were applied with either one following the other immediately, there was a sensitization of spores or mycelium, but if either treatment was applied 24 or 48 hours after the first one, a desensitizing effect occurred. Therefore, spores or mycelia, which were able to repair the heat or irradiation damage, were more resistant to subsequent treatments.

The post-irradiation recovery phenomenon has been

reported for Rhizopus stolonifer (107), when the sporangiospores were kept under conditions unfavourable to germination. Investigations with yeast (68) have shown that recovery after x-irradiation damage is an enzymatic process requiring a certain level of energy supply. The cytochrome system, pyridine co-enzyme and pyridoxal enzymes play an important role.

At 25 krad there was a slight increase in survival of mycelium when a heat treatment at 55°C followed irradiation after 24 hours (Fig. 47). This type of stimulation has also been reported for yeast (116), but no explantion was offered for this phenomenon.

The significance of the recovery phenomenon in spores and mycelia of A.flavus is obvious. If the two treatments of heat and irradiation are to be most effective in inactivating these structures, then they must be applied simultaneously or with a minimum of delay between them. Otherwise the cells will be able to repair the damage and thus produce a desensitizing rather than a sensitizing effect.

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