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RADIATION RESISTANCE OF ASPOROGENOUS BACTERIA IN FROZEN BEEF

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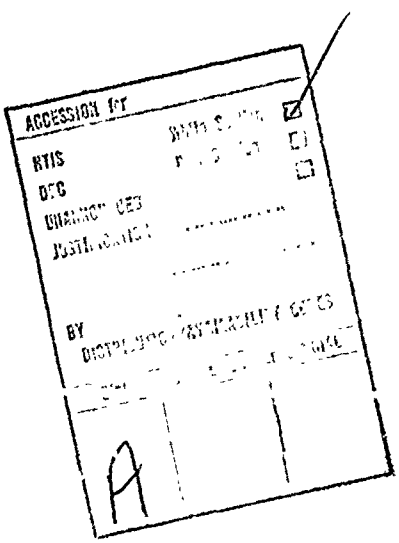

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A scheme was developed to isolate the most radiation resistant vegetative microbial cells occurring in beef. Selection of pure cultures and enrichment provided 16 apparently different isolates with higher radiation resistance than spores of Clostridium botulinum. Most of these bacteria were found to be Moraxella or Acinetobacter. They grew over a temperature range of 2° - 50°C. Preliminary data indicated the isolates to be relatively sensitive to heat and to limited oxygen. It would appear these organisms are rather widespread →

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and frequent in nature. Their significance, however, remains to be determined.

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INTRODUCTION

Irradiation is a potentially new technology for food processing. The primary objective in the application of high levels ($\geq$ 1 Mrad) of ionizing radiation is to make prepackaged food microbiologically stable without refrigeration. Thus, full microbial control must be attained.

In traditionally accepted processes of preservation, of all bacteria, the sporeforming ones are recognized to be the most resistant to biocidal processes. This general concept of resistance has been applied tacitly when exploring the applicability of irradiation to food preservation. The magnitude and relative resistance of vegetative cells and spores, however, has both a theoretical and practical interest in design and control of irradiation processes.

Vegetative cells vary greatly in their resistance to irradiation with a continuum of relative resistance from the sensitive pseudomonads (Maxcy and Tiwari, 1973) to the highly radiation resistant Micrococcus radiodurans (Anderson et al., 1956), Micrococcus radiophilus (Lewis, 1973), and some unusual "Moraxella-Acinetobacter" (Tiwari and Maxcy, 1972). The latter organisms were isolated from beef, but the literature in general indicated them to be rather widespread in nature, occurring in dairy products (Koburger et al., 1964), fish (Shewan, 1971), and vegetables (Snodgrass and Koburger, 1967). While these organisms are recognized to be relatively radiation resistant, no organized effort has been made to seek out the most radiation resistant vegetative bacteria in frozen (-30°C) beef and to compare their resistance to Clostridium botulinum spores, which is a common criterion for microbial safety.

The primary purpose of this work was to obtain the most radiation resistant

vegetative cells from a wide variety of samples of beef irradiated at -30°C and to determine their radiation resistance.

MATERIALS AND EXPERIMENTAL PROCEDURES

Culture treatments

The plating and counting procedures for determining microbial numbers were those described in "Recommended Methods for the Microbiological Examination of Foods" (APHA, 1966). The culture and identification techniques were those described in "Manual of Clinical Microbiology" (Tatum et al., 1974) amd Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

Spores of Clostridium botulinum were produced biphasically in the Natick Development Center according to the methods of Anellis et al. (1972).

To obtain the full spectrum of potential contaminants of beef, samples were taken to represent numerous sources. A commissary buying from four slaughter operations, a supermarket with a central processing operation buying from various packers, an independent supermarket with known low quality, and frozen beef from Mexico were the sources. Sampling plans included cold weather months and warm weather months for each source. These samples were commonly in the form of ground products suitable for hamburger. From the time the beef was obtained at the commercial source, aseptic techniques were used.

Irradiation was with a $Cobalt_{60}$ source as described by Tenny and Miyauchi (1970) providing a dose rate of approximately 10 Krad per minute. Temperature control for frozen samples was obtained by placing dry ice near the sample with appropriate insulation. Where the temperature was indicated as -30°C the range was ±10° C. The temperature was monitored by means of a thermocouple and

appropriate recorder.

When vacuum packaging was employed, sealing was at 125 mm mercury pressure.

Isolation procedure

Samples of ground beef were packaged in flexible polyethylene pouches and irradiated at -30°C. Preliminary experiments were performed to determine the dose level required to reduce the count so that a 1:10 dilution gave approximately 10 colonies per plate in the counting procedure. A 1 Mrad dose was found to be appropriate and was used in subsequent isolation and enrichment procedures. There were five factors considered in this scheme: 1. Samples were packed in air and sealed before irradiation. 2. Samples were vacuum packed and sealed before irradiation. 3. Samples packed in air for irradiation were subsequently plated and incubated aerobically. 4. Samples irradiated in vacuum packages were plated and incubated aerobically. 5. Samples irradiated in vacuum packages were plated and then incubated under anaerobic conditions. All the above plating was on plate count agar. The colonies arising from the various platings were subcultured and observed for differences in morphology, catalase production, proteolysis reaction in litmus milk, and clonal characteristics. A culture differing in any characteristic from other isolates was saved for further study. The processes were repeated for various sources of meat. Approximately 60 cultures were chosen for further observations.

The above isolates were grown in pure cultures in m-plate count broth (Difco) until the broth was turbid and contained approximately 10^9 cells per milliliter. One milliliter of each of 6-8 cultures was combined and from this mixture, one milliliter was added to approximately 1 lb of ground beef previously given a 200 Krad dose. The 200 Krad dose was to reduce the numbers of sensitive microorganisms

while those resistant cells naturally occurring in beef were included along with the inocula to be studied for the most radiation resistant isolates. Admixture was made by regrinding in a food chopper. The final product contained approximately 250,000 microbial colony forming units per gram. The inoculated product was then irradiated with 1 Mrad to destroy bacteria to the extent of allowing approximately 10-300 colonies per plate based on a 1:10 dilution. This treatment was considered as the second enrichment from which 16 apparently different non-sporeforming cultures were obtained. Criteria of difference also included the phenomenon of radiation resistance.

Relative resistance

To determine radiation resistance, pure cultures were grown to their maximum stationary phase at 32°C in m-plate count broth using a shaker incubator unless growth was rapid in quiescent incubation. Each culture was inoculated into ground beef previously irradiated at 2 Mrad (except where selective media were used and will be so indicated in the "Results" section) to provide a test population of approximately 10^6-10^7 cells per gram. The samples were mixed by repeated grinding, formed into patties, placed into flexible pouches, sealed under vacuum, frozen, held overnight, and irradiated at -30°C. The irradiation dose levels were chosen to produce a 4-5 log cycle reduction in the inoculum. The exact dose levels were dependent on the relative resistance of the isolate being studied and are reflected in the results presented in the next section.

Relative heat resistance was determined by growing the isolates in m-plate count broth to their maximum stationary phase, which was a population density of approximately 10^9 per ml. The cultures were then subjected in broth in which they had been grown to various time-temperature treatments to obtain a minimum 4 log

cycle reduction in the population density. It was necessary to determine heat resistance at temperatures from 55°C to 70°C, because of the great variation in heat resistance.

Isolates representing low, intermediate, and high heat resistance were studied further. A pure culture was mixed into beef previously irradiated with a 2 Mrad dose. The inoculated beef was then placed into polyethylene pouches and flattened to a sample thickness of 3 mm. The temperature of the center of a control pattie was monitored with a thermocouple and a recorder.

Characterization of isolates

Each of the isolates was able to grow under aerobic conditions. To obtain abundant growth, however, some isolates required a shaker incubator. Some isolates grew in simple nutrient broth while others were more fastidious, e.g., they grew well in m-plate count broth but relatively poorly in trypticase soy broth. Early work was hampered somewhat by the unexpected inability of certain isolates to grow well in trypticase soy broth. This difficulty was overcome by applying the findings of Anellis (personal communication) that 100 fold more growth could be obtained by substituting m-plate count broth for growth of pure cultures for subsequent inoculation processes.

For the detailed characterization and classification, Gram stains were prepared from cultures on plate count agar and in m-plate count broth previously incubated for 16-24 hr at 32 C and 37 C and examined for microscopic morphology. Isolated colonies on plate count agar, trypticase soy agar, trypticase soy agar containing 5% defibrinated sheep blood, trypticase soy agar with 5% bovine serum, chocolate blood agar with 5% sheep blood and brain heart infusion agar containing 5% defibrinated rabbit blood were observed periodically from 16 hr to 7 days.

Tests and media used to determine physiological characteristics were: oxidase reaction; catalase production; motility (microscopic examination of a hanging drop preparation, darkfield, motility test agar and SIM agar); reactions on triple sugar iron agar; oxidation fermentation of glucose (Hugh and Leifson, 1953, O-F basal medium containing 1% glucose, cystine trypticase agar with 1% glucose, and phenol red agar base with 1% glucose); nitrate reduction (nitrate broth); gelatin hydrolysis (nutrient gelatin); indol production (1% tryptone broth and SIM agar); litmus milk; citrate utilization (Koser's citrate); urease activity (urea broth); and phenylalanine deaminase activity.

Presence or absence of growth on basal mineral medium, O-F without carbohydrate, MacConkey agar, eosin methylene blue agar, Shigella-Salmonella agar, and growth on plate count agar with 2.5% and 6.5% NaCl was observed. Penicillin susceptibility was tested by the technique of Bauer et al. (5) using 10 unit discs. Thermal limits of growth were determined by culturing bacteria on trypticase soy agar and incubating for up to 7 days at temperatures ranging from 0°C to 50°C. An anionic surfactant, "Ultrawet" (Atlantic Refining Co., Chicago, Illinois), was incorporated into trypticase soy agar and trypticase soy broth at concentrations of 0.01, 0.1 and 1.0% in order to test susceptibility to surfactant.

RESULTS

Selection of radiation resistant isolates

Sources of beef and sampling procedures were chosen to include all the normally expected microbial contaminants in beef. Packing in vacuum and in air for irradiation provided a normally expected environment as well as the environment of chance mechanical failure in packaging operations. Incubation for

recovering the surviving bacteria involved both aerobic and anaerobic procedures. Most of these isolates were highly aerobic and none required anaerobic incubation for growth. Thus, the enrichment and isolation procedures provided pure cultures of the most radiation resistant vegetative cells occurring in beef. A total of 16 isolates that appeared to be different by screening procedures in pure culture were studied further for characterization.

Radiation resistance of isolates

The radiation resistance at -30°C of each isolate vacuum packed in beef was determined by aerobic plate counts of the surviving bacteria. Preliminary runs were made to determine the general pattern of destruction and dose requirements to obtain a usable death curve. Then at least two separate runs were made for data to determine the rate of destruction. Typical death curves are given for isolates with high (Figures 1, 2) and intermediate (Figure 3) resistance. All survivor curves similar to that shown in Fig. 3 were assumed to be a linear regression and the slope of the line was calculated by the method of least squares (Sokal and Rohlf, 1969). Conforming to the definition of D_{10} as the reduction of the population by one log cycle, the values expressed in Krad are shown in Table 1.

Those isolates with a high resistance to radiation (Figures 1, 2) required a disproportionately high dose for the first decimal reduction of the cell population, which resulted in a major "shoulder" on the semi-logarithmic plot of the survival curve. The destruction rate was therefore presented in two different forms (Table 2). The first decimal reduction was determined graphically. The final rate of destruction was estimated by the method of least squares using

only those points that appeared on the graph to be beyond the shoulder.

A number of trials were made with the various isolates with the same procedure to study radiation destruction as given above, except that the packages were not sealed under vacuum. The cells were equally or more sensitive when irradiated in air packs than when irradiated in vacuum packs.

In the early stages of this work it was apparent that isolate #4 was one of the most radiation resistant; therefore it was used for comparative and exploratory purposes. A comparison was made between the radiation resistance at -30°C and +30°C in m-plate count broth. The cells were considerably more resistant at the lower temperature (Figure 4).

Identification of isolates

The results of the various observations to characterize the isolates were used to place them into groups and to give generic and species names to some. Data on radiation resistance and heat resistance are given in Table 1. A detailed report of characterization of these isolates has been presented (Welch and Maxcy, 1975).

Radiation resistance of C. botulinum spores as compared to isolate "4"

The radiation-resistance of C. botulinum type 33A spores in beef was determined at -30°C using the vacuum packaged preparation system previously described. The numbers of survivors were determined by the agar tube method. C. botulinum spores were considerably less radiation-resistant than isolates #4 and 7 (Figure 1, Table 2).

Heat resistance of the radiation resistant isolates

For comparative purposes and to direct further work, the heat resistance of pure cultures of the individual isolates was determined in m-plate count broth in which they had grown. There was considerable variation in the heat resistance as shown by the data in Table 1. The D_{10} values ranged from 2.5 min at 55°C to 5.4 min at 70°C.

The above work was exploratory to be used as a guide for further work with meat. Preliminary data indicated the isolates were generally more resistant in beef than in broth.

Range of growth temperatures

The minimum and maximum temperatures for growth of the isolates on trypticase soy agar were determined. For the group as a whole, the range was from 2°C to 50°C. The greatest range for an individual isolate was 5°C to 50°C (Table 3).

Relative oxygen demands for growth of the radiation resistant isolates

Those isolates that had been considered different by morphological and biochemical tests were taken from growing liquid cultures and streaked on plate count agar. Wedges of the agar with the individual cultures were vacuum packed in flexible pouches and incubated at 32°C. Control samples were packaged without a vacuum treatment. Visual comparisons were made to determine the relative growth response. While each of the isolates could tolerate the limited oxygen environment to some extent, four were considered most tolerant and were studied further to determine their fate in vacuum packed ground beef.

Fresh ground beef was irradiated with 2 Mrad at 30°C, which provided beef with less than 10 microbial colony forming units per gram. Beef was then inoculated with isolates to be studied. Results with an isolate apparently tolerant to reduced oxygen and radiation resistant are given in Figure 5. The samples were incubated at 32°C with periodic determination of numbers of microorganisms by plate counts. The results indicated the isolates were able to grownin the reduced oxygen packages but at a less rapid rate than in the air packs (Figure 5).

Radiation resistance of some vegetative cells of public health interest

To determine the radiation resistance of some vegetative cells of public health interest, Staphylococcus aureus, Salmonella enteritidis, and Escherichia coli were used. Cultures were grown to the maximum stationary phase for use as an inoculum. Ground beef containing an average plate count of 79,000 was irradiated with 200 Krad at 30°C, which reduced the average total plate count to 450 per gram. Counts with selective media showed that the residual flora consisted of less than 1 per gram of the type organisms being studied in this experimental series. The inoculum and beef were mixed and packaged under vacuum, frozen, and irradiated at -30°C. Numbers of a surviving inoculum were determined by plating on selective media as follows: Staphylococcus Medium No. 110 for S. aureus; Violet Red Bile Agar for E. coli; and Bismuth Sulfite Agar for S. enteritidis. Based on the average of three trials with each organism the D_{10} values were 95 Krad for S. aureus, 59 Krad for E. coli, and 107 Krad for S. enteritidis.

Comparative radiation destruction was also made using four strains of Streptococcus faecium with an irradiation treatment at -30°C in broth. The D_{10} values ranged from 141-182 Krad, which indicated more sensitivity than the results reported by Anellis et al. (1973) using an irradiation temperature of -80°C.

DISCUSSION AND CONCLUSIONS

The preceding data indicate that beef contains asporogenous vegetative bacteria which are highly radiation resistant at -30°C. These bacteria belong to the genera Acinetobacter and Moraxella or would be closely related in any classification system. Though the frequency of occurrence of the highly resistant forms of these bacteria is not known, the literature indicates the genera Moraxella and Acinetobacter to be widely distributed in nature and to be associated with various food products. Tiwari and Maxcy (1972) found that these genera accounted for up to 42% of the total microflora of commercial ground beef. It is not possible, however, to equate numbers as reported by their work and numbers of those cells highly radiation resistant at -30 C found in the work of this report.

While these isolates of the genera Moraxella and Acinetobacter were more radiation resistant than spores were, there was not a comparable resistance to such other limiting factors as heat and oxygen. Perhaps the sensitivity to factors other than radiation explains the lack of previous encounter and recognition during extensive work with irradiation sterilization using C. botulinum as the test organism.

Expressions of radiation resistance of a culture can be simple and precise if there is a straight line relationship between logarithm of surviving population and the applied dose. This relationship is well established for most

radiation sensitive bacteria. Certain resistant microorganisms such as spores of C. botulinum and vegetative cells of isolates #4 and #7 of this work, however, show disproportionately high resistance in the early stages of the death curve. Thus, traditional methods of calculation of death rate and expression of a D_{10} value for these organisms constitute only an approximation of a relative value. There are tremendous differences in values depending on what part of the death curve is chosen for calculations (Table 2). Additional work in this area is needed to establish a system to express the destruction of a population or the probability of a survivor after various doses.

In light of the above anomalies in death curves, it is apparent that different reports may present different D_{10} values. When Lewis (1971) reported isolating a highly radiation resistant Micrococcus sp. the radiation dose was limited to 1600 Krad. Had he extended the dose to a higher level, the D_{10} value likely would have been less. Caution is warranted against attaching too much significance to absolute D_{10} values until a better system of calculation is developed.

In taking a long range view of the applicability of irradiation processing of food, the role and significance of the Moraxella and Acinetobacter will need further delineation. As a first step, a selective test to study sources, monitor channels of contamination, and determine survival would be most helpful. Their significance in relation to the total flora then could better be determined.

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TABLE 1. Radiation and heat resistance of asporogenous bacteria isolated from fresh beef

Classification	Isolate Number	Radiation D_{10} in Krad	Heat Resistance D_{10} in Minutes	Heat Resistance Temperature of Heating (°C)
Moraxella nonliquefaciens	3	583	5.2	55
	D	539	2.9	60
Moraxella osloensis	5	1000	1.5	60
	17	671	---	--
	A	582	5.5	60
	K	764	2.5	63
	N	477	3.0	60
M-5	4	See Table 2	5.4	70
	7	" " "	7.8	65
Acinetobacter calcoaceticus	1	814	6.7	65
	E	405	6.3	65
	I	591	4.5	65
Intermediates[a]	H	480	5.8	60
	J	273	5.6	60
Brevibacterium	2	485	2.5	55
	9	642	2.8	55

[a] Characteristics of these organisms did not fit with recognized groups.

TABLE 2. Comparative radiation resistance of selected isolates and C. botulinum 33A spores

Organism	Isolate Number	D_{10} values (Krad)	
		First Decimal Destruction	Final Destruction Phase
M5	4	2230	628
	7	4000	452
C. botulinum 33A spores	-	975	294

TABLE 3. Range of growth temperatures of some bacteria isolated from fresh beef

Classification	Isolate Number	Temperatures (°C) 0	2	5	10	20	32	37	42	45	50
Moraxella nonliquefaciens	3		———	———	———	———	———	———			
	D		———	———	———	———	———	———			
Moraxella osloensis	5		———	———	———	———	———	———			
	17			———	———	———	———	———			
	A			———	———	———	———	———			
	K				———	———	———	———	———		
M-5	4			———	———	———	———	———			
	7					———	———	———			
Acinetobacter calcoaceticus	1					———	———				
	E			———	———	———	———	———	———	———	———
	I					———	———	———			
Intermediates[a]	H			———	———	———					
	J			———	———	———	———	———	———	———	
Brevibacterium	2			———	———	———	———	———			
	0					———	———	———			

[a]Characteristics of these organisms did not fit with recognized groups.

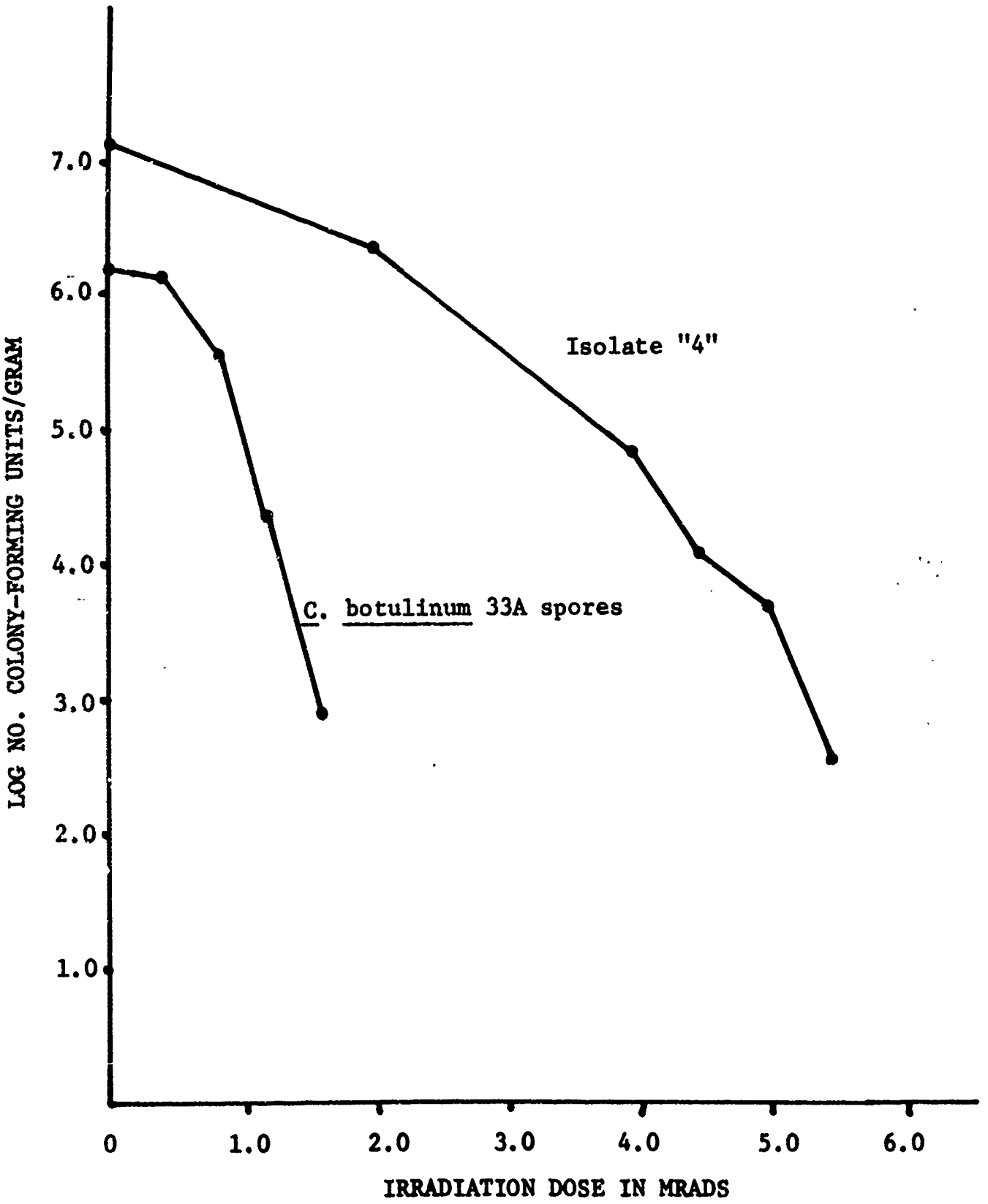

Figure 1. Comparative radiation resistance of C. botulinum 33A spores and Isolate "4" in ground beef at -30°C.

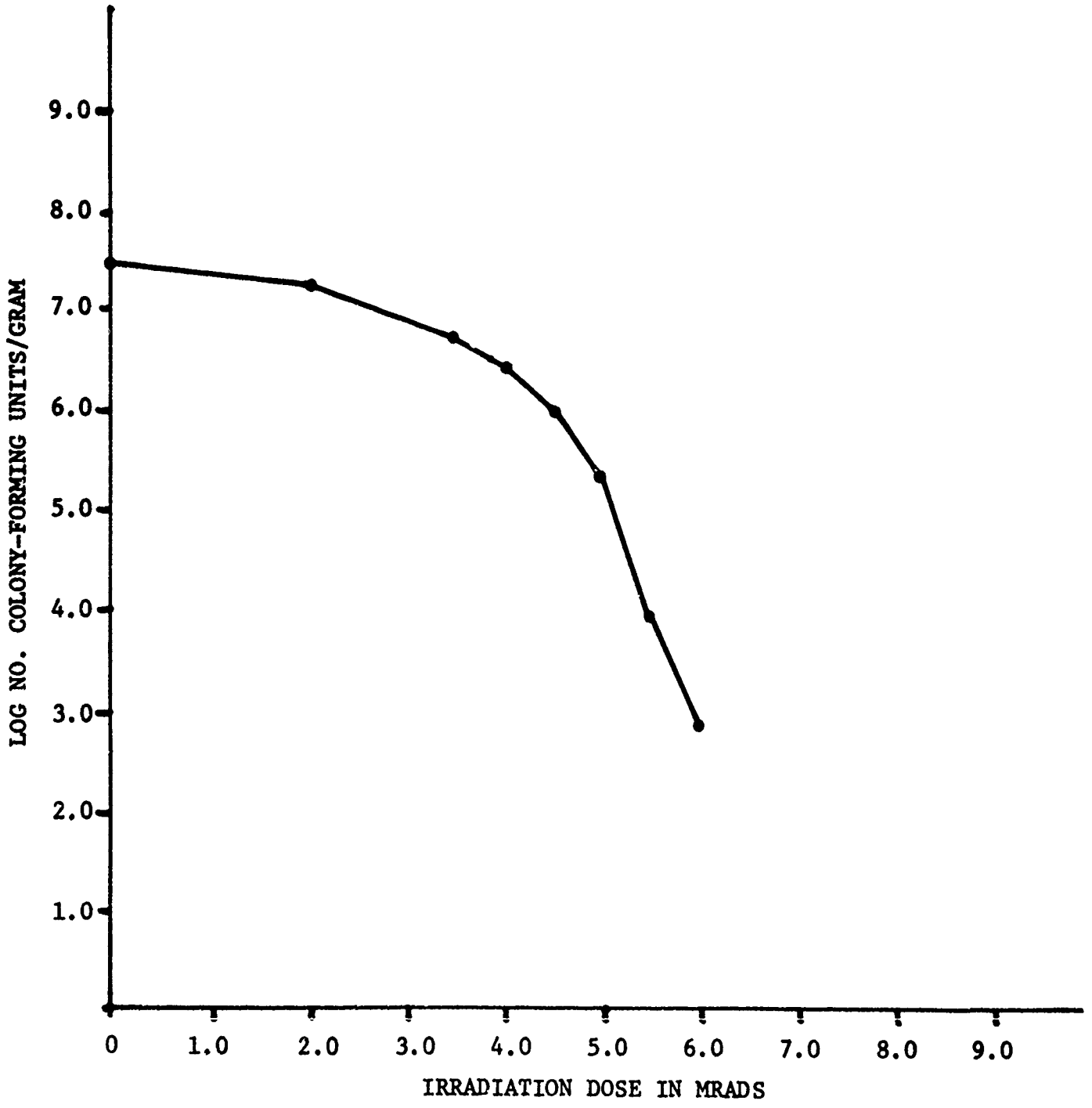

Figure 2. Radiation resistance of Isolate "7" in ground beef at -30°C.

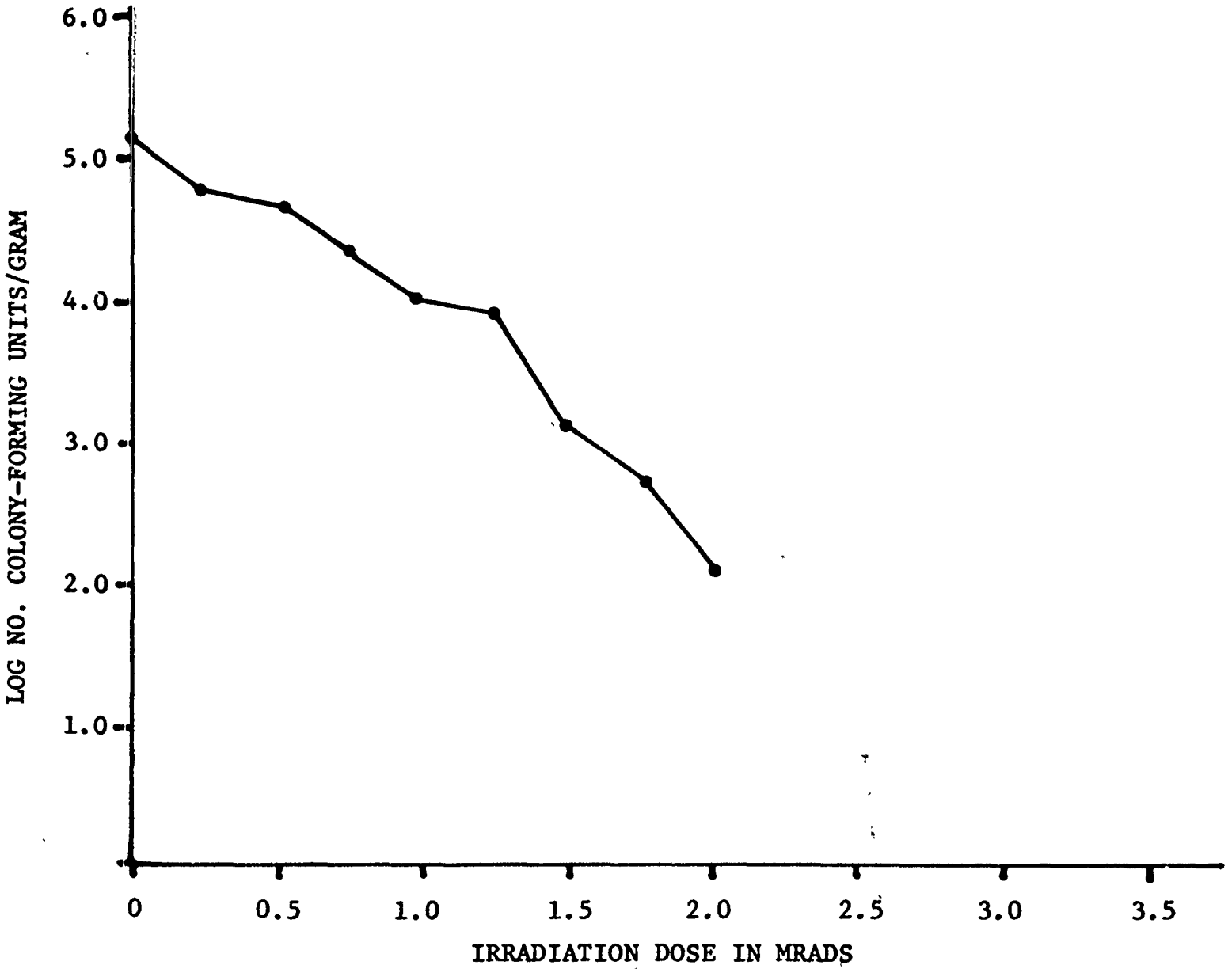

Figure 3. Radiation resistance of Isolate "17" in ground beef at -30°C.

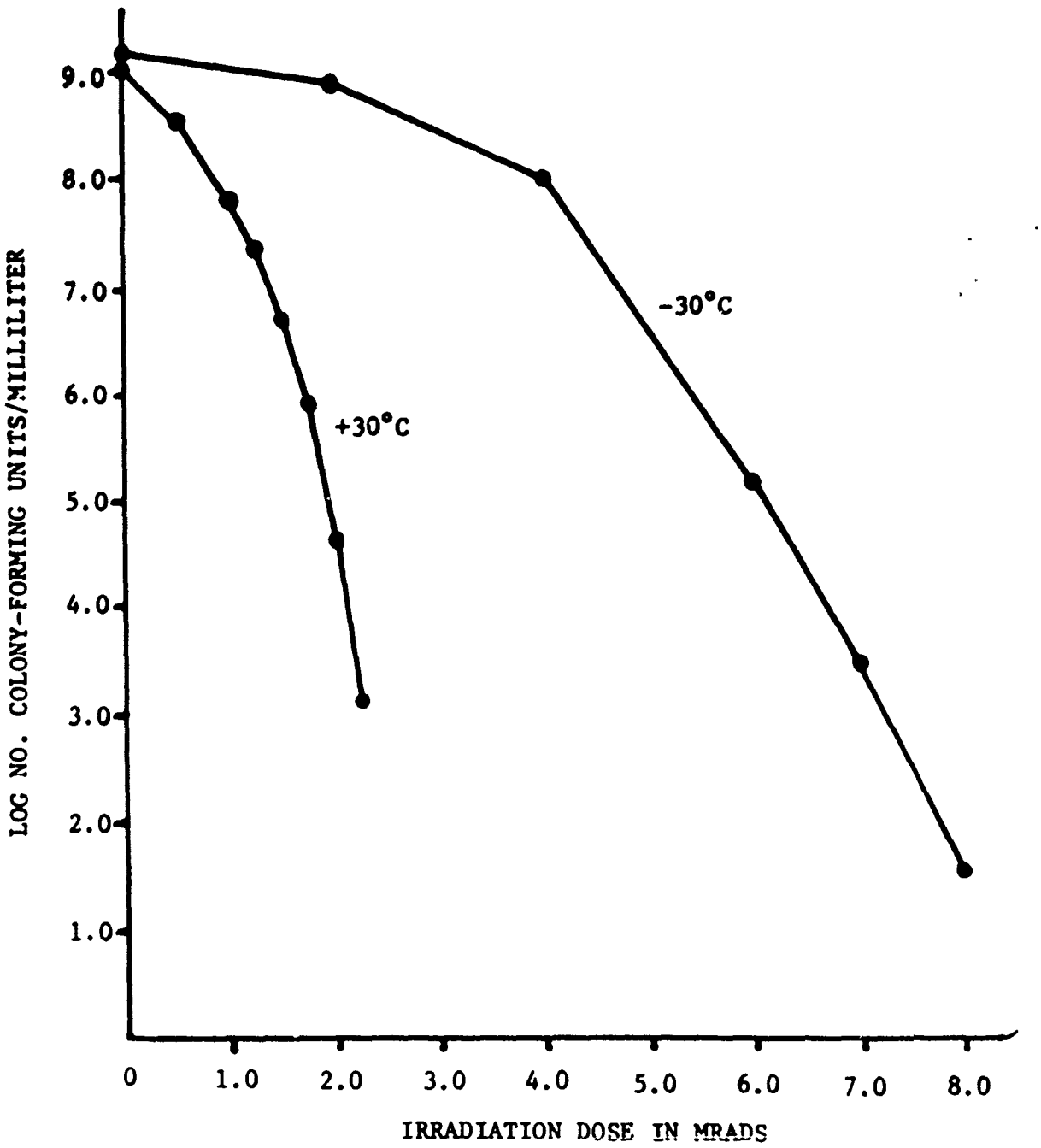

Figure 4. Comparative radiation resistance of Isolate "4" in m-Plate Count Broth at +30°C and -30°C.

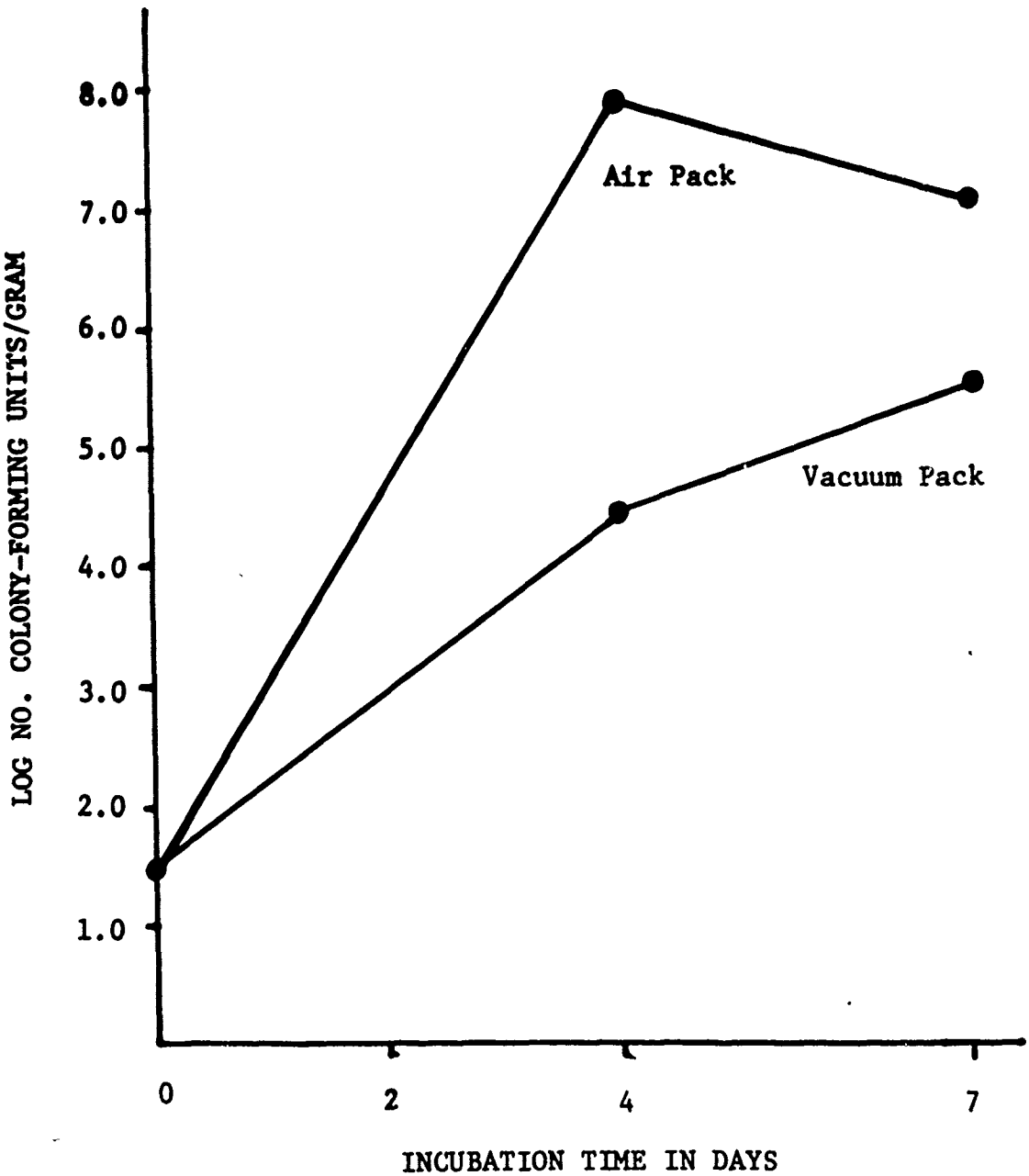

Figure 5. Comparative growth at 32°C of Isolate "H" inoculated into radappertized ground beef in air pack and vacuum pack.