T H E   U N I V E R S I T Y   O F   M I C H I G A N

COLLEGE OF ENGINEERING
Department of Chemical and Metallurgical Engineering

Progress Report

A STUDY OF THE EFFECT OF IONIZING RADIATION ON RESISTANCE,

GERMINATION, AND TOXIN SYNTHESIS OF CLOSTRIDIUM

BOTULINUM SPORES, TYPES A, B, AND E

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SUMMARY

Human botulism has been reported to result from eating fish and seafood products in which Clostridium botulinum Types A, B and E have grown. With one exception, all cases of Type E botulism have been attributed to food products from the sea.

Since the compilation of our review presented in the first progress report, two additional outbreaks of Type E botulism have come to our attention. One occurred in Sweden in November, 1960, and was due to pickled herring. The other outbreak, which led to two fatalities, occurred in Detroit in March, 1963, and was due to consumption of commercially canned tuna fish. The latter outbreak is of particular significance, since it was not only due to C. botulinum Type E, but also represents the first case of botulism due to a commercially canned product in the United States in 25 years.

The outbreak in Sweden prompted a study of the distribution of the organisms in nature. Johanssen[1] has isolated the organisms from samples collected along the coastline of Sweden, and from potato peelings.

In the present study six representative strains of C. botulinum Type E are being used. Additional strains have been obtained from Japan, Sweden, and Denmark. These strains will be examined in order to determine whether they possess marked physiological differences from the six that we are now studying.

Growth and toxin production in haddock homogenates and broth were studied with incubation at temperatures between 0-37°C. At 37.5°C, 33.0°C, and 18.0°C, growth occurred within three days. At 8.3°C, and with some strains at 5.4°C and 3.3°C, growth as measured by gas production was variable. However, low levels of toxin were detected in the samples within 38 days of incubation. At 0°C, no growth has occurred in any of the samples after 138 days of incubation.

Activation of the cultures with trypsin was necessary in order to detect low levels of toxin in the filtrates.

Resistance of the spores of the six strains of Type E to gamma radiation in phosphate buffer was found to be similar. Irradiation of the spores in haddock homogenate and oyster juice resulted in an increase in survivors which was 100-1000 greater than the increase resulting from irradiation under similar conditions in buffer.

Toxin synthesis as influenced by media composition and variation is being studied. No toxin was detected in oyster juice which had been previously sterilized with gamma radiation, inoculated with Type E spores, and incubated at 33°C, although toxin was detected in similar experiments using oyster juice that had been both irradiated and heated. With haddock homogenate, toxin was produced both in the radiation sterilized media and in the irradiated and heated media when the media were inoculated with Type E spores and incubated. Activation of the preformed toxin in tissue homogenates was variable. This technique needs further study, however.

Effects of carbohydrates and of the pH of the medium are also being studied. Variation of pH in the range of 6.5-7.5 had little effect on the production of toxin, although growth was slightly heavier at the higher pH. Incubation at this pH also resulted in a slight increase in the amount of toxin recovered. As long as a fermentable carbohydrate was present, growth and toxin synthesis occurred. In future experiments, better correlation of the extent of growth and toxin production will be sought.

The afore-mentioned studies are being continued and extended. The low temperature incubation studies are being extended to include more substrates, in order to test the effects of incubation temperatures and substrate variation on growth and toxin production.

The radiation resistance of the spores of one strain of Clostridium perfringens Type A, has been determined in phosphate buffer. This bacterium will be studied further, using other food poisoning strains and suspending media.

Dose rate measurements about the two cobalt-60 sources available for this study have been completed.

INTRODUCTION

Microbiological parameters are of primary importance in evaluating the
wholesomeness, refrigerated storage qualities and safety of radiation pas-
teurized seafoods. Relevant to the safety of the foods are the toxigenic
anaerobes Clostridium botulinum and Clostridium perfringens. In the case of
C. botulinum, the three types A, B and E have been associated with food
poisoning from eating contaminated seafoods. With a single exception, all
outbreaks due to C. botulinum Type E in the United States have been due to
the consumption of fish, fish products and sea mammals have been caused by
one of the three types of C. botulinum Type E. Because of the ability of
Type E spores to germinate and produce toxic cultures at refrigerator tem-
peratures, consideration must be given to this group of organisms in the
evaluation of processes used to prolong the storage life of seafoods at
low temperatures.

The broad objectives of the present study are to gather information
on the germination, growth and sporulation of representative strains of
Type E, the factors which govern the synthesis of their toxin and the re-
sistance of these spores to radiation and heat. Specifically, the germin-
ation and outgrowth of Type E spores is being studied under optimal condi-
tions and in fish and fish products in the temperature range of 0-03°C.
Concomitantly, the rate and extent of toxin synthesis must be considered
under the same conditions. The radiation resistance and factors affecting
the resistance of the spores to radiation need study to ascertain the
effect of irradiation on the subsequent germination and outgrowth of the
spores. Particular emphasis is being placed on strains of C. botulinum
Type E spores, but Types A and B spores are also used where applicable.

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OBJECTIVES

Our objective in the present experiments is to determine the minimum temperature and conditions under which the spores of C. botulinum Type E will germinate, develop cultures and produce toxin. Concurrently, the influence of such medium variations as pH, sugar and nitrogen sources on toxin production is being studied. The radiation resistances of the spores of the six Type E strains, of Types A and B, and of C. perfringens are being tested and compared.

THE EFFECT OF TEMPERATURE ON GROWTH AND TOXIN PRODUCTION

The rates of growth and toxin development are being studied in three media and at seven incubation temperatures between 0-37°C (32-98.6°F) using six strains of C. botulinum Type E.

Procedures

Media.—The three media currently employed in the study are haddock homogenate, beef infusion and trypticase peptone broth.

The haddock homogenate was prepared by blending 1 part of frozen haddock fillets (Newfoundland) with two parts of distilled water in a Servall Omni-Mixer. The homogenate was filtered at 2°C. Part of the tissue pulp was dispensed into 20x150 mm (25 ml) screw cap tubes and then filled with filtrate. The tubes were immediately sterilized with 3 megarads of gamma radiation. The pH of the haddock homogenate was 7.2.

The composition of the broth media was as follows:

<u>Beef Infusion Broth (BI)</u>

Beef Infusion*	1 liter
Bacto Peptone	1%
Di-Sodium Phosphate	0.2%
Glucose	0.2%
Sodium Chloride	0.5%
Sodium Thioglycollate	0.1%
Dried Meat Particles	
pH 7.2-7.4	

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*1 pound of beef/liter distilled water, boiled 1/2 hour, chilled, filtered.

<u>Trypticase</u> <u>Peptone</u> <u>Broth</u> <u>(TP)</u>

BBL Trypticase	5%
Bacto-Peptone	0.5%
Sodium Thioglycollate	0.1%
Glucose*	0.2%
pH 7.2-7.4	

The broth media was contained in 16x150 mm screw cap tubes.

<u>Inoculum.</u>—The six Type E strains used in this experiment were Vancouver Herring, Seattle Forks, Beluga, E-74, Nanaimo and Iwanai. Spores of the organisms to be used for inocula were grown in 200 ml trypticase peptone broth at 33°C for 36 hours. The spores were washed six times in cold distilled water and stored in distilled water under refrigeration. Viable counts of the stock suspensions were made in beef infusion agar (see Table I).

TABLE I

VIABLE COUNTS OF STOCK SPORE SUSPENSIONS
OF <u>C</u>. <u>botulinum</u>, TYPE E

Strain	Count* (Spores/ml)
Nanaimo	8.0×10^8
Vancouver Herring	2.7×10^9
Seattle Forks	4.7×10^9
Beluga	4.3×10^9
E-74	1.3×10^9
Iwanai	3.0×10^8

*Pre-heated 10 minutes at 65°C.

For inoculation of the culture tubes an aliquot of the stock suspension was heated and detoxified at 65°C for 10 minutes. The suspension was diluted 1000-fold in sterile distilled water to produce a final concentration of 10^6 spores per ml. One ml of the spore suspension was used to inoculate tubes of haddock homogenate, while 0.1 ml was used for the same purpose in the tubes of broth. Immediately after inoculation, all tubes were layered with 3% agar

*Added after sterilization.

containing 0.4% sodium thioglycollate. During the procedure of inoculation, all tubes were kept cold in an ice water bath. After inoculation, the tubes were placed in the incubators. Four tubes of haddock homogenate and beef infusion were inoculated per strain of organism for each incubation temperature. One tube of clear TP media was inculated with each strain in order to observe changes in turbidity during growth.

Incubation.—The culture tubes were incubated at the following temperatures: 37.5°C (99.5°F), 33.0°C (91.5°F), 18.0°C (64.4°F), 8.3°C (46.9°F), 5.4°C (41.8°F), 3.3°C (38.0°F) and 0.0°C (32.0°F), ± 0.5°C (0.9°F).

BOD-type incubators were used for the lower temperatures.

In order to minimize temperature fluctuations in the culture tubes when the incubator doors were opened, the tubes were placed in a water bath. Temperatures were monitored by placing two thermocouples in a blank culture tube, one on the top and one on the bottom of the tube. The thermocouples were connected to both a manual and a recording potentiometer. Under these conditions, the temperatures within the incubators were found to vary between ± 0.5°C (0.9°F). The low temperature incubators for the cultures are shown in Fig. 1.

Growth and Toxin Assay.—Growth was ascertained by the observation of gas bubbles collecting beneath the agar layers, by the development of off-orders and by clearance of the opalescence of the homogenates. Confirmation of growth was established by the presence of toxin. Where criteria of growth were absent, toxin assay alone was used.

For toxin determination in the cultures, the contents of the tubes were mixed and centrifuged to remove suspended particles. Mice were injected intraperitoneally with 0.5 ml of the supernatant fluid. Toxin was checked with homologous anti-toxin. Titres of toxin were determined by making serial dilutions of the toxin in gelatin-phosphate buffer.

Samples which gave low titres or no deaths were treated with 0.1% trypsin at 37°C for 1-2 hours at pH 5.8-6.0.

Results

At the incubation temperatures of 37.5°C and 33°C, cultures developed gas in all culture tubes within 24 hours, whereas at 18°C; the average time required was 2-3 days. All of the strains but one produced cultures in haddock homogenates within 9-12 days at 8.3°C; in broth, cultures developed 1-2 days earlier at this temperature. Two strains, the Iwanai and Vancouver Herring, have not developed gas in haddock homogenates after 138 days of incubation at 5.4°C; the other four strains developed gas within 15 days.

Fig. 1. Incubators used for low temperature growth studies of bacterial cultures.

In broth, all the strains developed gas at this temperature within 138 days, but the time of gas production varied with the different strains. At 3.3°C, only one tube out of four inoculated with spores of strain E-74 showed definite gas production. The Beluga strain grew in beef infusion and trypticase broth in 20 days; the Seattle Forks strain grew in beef infusion in 12 days. At 0°C no growth has become evident in any strain after 138 days of incubation. Data for the growth experiment are summarized in Fig. 2 and Table II.

TABLE II

TIME FOR GAS FORMATION BY C. botulinum IN VARIOUS

MEDIA AND AT VARIOUS TEMPERATURES

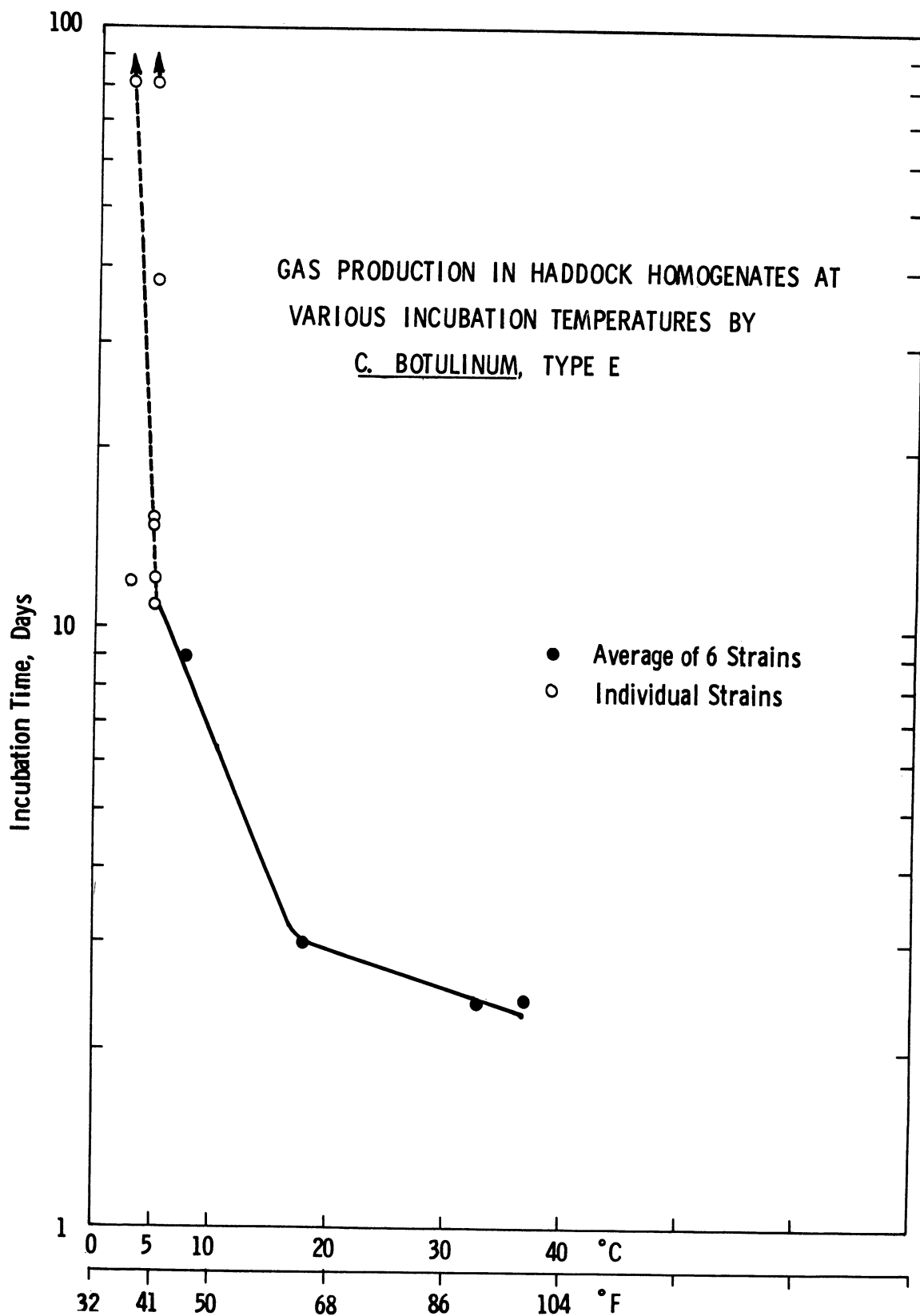| Type E Strain | Incubation Temperature | | Time to Develop Growth | | | | | |
| | | | Haddock Homogenate | | Trypticase Peptone | | Beef Infusion | |
	°C	°F	hr	days	hr	days	hr	days
Nanaimo	37.5	99.5	48		<24		<24	
	33.0	91.5			<24		<24	
	18.0	64.4	72					
	8.3	46.9		10		12		
	5.4	41.8		12		38		50
	3.3	38.0		>138		>138		>138
	0.0	32.0		>138		>138		>138
Vancouver Herring	27.5	99.5	48		<24		<24	
	33.0	91.5	48		<24		<24	
	18.0	64.4	72		48		48	
	8.3	46.9		12		12		9
	5.4	41.8		>138		>50		>50
	3.3	38.0		>138		>138		>138
	0.0	32.0		>138		>138		>138
Seattle Forks	37.5	99.5	48		<24		<24	
	33.0	91.5	48		<24		<24	
	18.0	64.4	96		48		48	
	8.3	46.9		12		8		7
	5.4	41.8		15		38		12
	3.3	38.0		>138		>138		138
	0.0	32.0		>138		>138		>138
Beluga	37.5	99.5			<24		<24	
	33.0	91.5	48		<24		<24	
	18.0	64.4	72				48	
	8.3	46.9		10		8		7
	5.4	41.8		15		21		12
	3.3	38.0		>138		20		20
	0.0	32.0		>138		>138		>138
Iwanai	37.5	99.5	48		<24		<24	
	33.0	91.6	48		<24		24	
	18.0	64.4	96		48			
	8.3	46.9		>138		12		12
	5.4	41.8		>138		38		>50
	3.3	38.0		>138		>138		>138
	0.0	32.0		>138		>138		>138
E-74	37.5	99.5	48		<24		<24	
	33.0	91.5	48		<24		<24	
	18.0	64.4	72		48		48	
	8.3	46.9		9		10		7
	5.4	41.8		11		12		12
	3.3	38.0		12		>138		>138
	0.0	32.0		>138		>138		>138

Fig. 2. Gas production in haddock homogenates at various incubation temperatures by C. botulinum Type E.

The results of the toxin assays performed on the cultures developed in haddock homogenates are summarized in Fig. 3. Except with strain E-74, no toxin was detected in the haddock homogenates after two days of incubation at 37°C. Maximum titres occurred at 18°C, being of the order of 200-20,000 MLD per ml, without activation. At 5°C low titres, 2 MLD, of toxin were obtained after 38 days of incubation. The titres were increased to 8-16 MLD by activation with trypsin. At 3.3°C no toxin was detected in any strain until activation with trypsin after 38 days incubation; then low titres, 4 MLD, were yielded. At 0°C no toxin was detected after 80 days incubation.

Discussion

It was observed that low levels of toxin may be present even though outward manifestations of growth, such as gas production in the cultures, are absent. Furthermore, samples that are suspected of containing toxin must be activated with trypsin before the absence of toxin can be established. In our studies, however, activation or potentiation of the performed toxin in haddock homogenates and other seafood substrates has been variable. In some cases, titres were increased slightly; in a few cases, actual decreases in titres occurred. This procedure needs further investigation. Since tissue enzymes are not inactivated by gamma radiation, such enzymes may conceivably potentiate the toxin precursor or degrade the toxin, or even accomplish both concurrently.

OTHER GROWTH STUDIES

The turbidity of media containing tissue particles makes it difficult to follow the parameters of growth, such as the length of the lag phase and the extent of growth. Another method being employed in our studies is that of following turbidity changes in clear broth cultures in order to obtain corollary information.

Results of an experiment in which spores of E-74 were inoculated in trypticase peptone broth and incubated at various temperatures are presented in Fig. 4. In this experiment approximately 10^5 spores were inoculated into 7 ml of trypticase peptone broth (containing 0.2% glucose) contained in screw cap tubes. One tube was withdrawn from incubation at the time intervals indicated, and turbidity was measured.

The rate of growth was greatest at 37°C, but the extent of growth was greatest at 33°C, which is the approximate optimum temperature for these organisms. There was a progressively greater lag and lessened growth as the temperature was lowered. At 5°C very little turbidity developed.

TOXIN TITRES FOR 6 STRAINS OF
C. BOTULINUM TYPE E CULTURES IN
HADDOCK HOMOGENATES AT VARIOUS TEMPERATURES
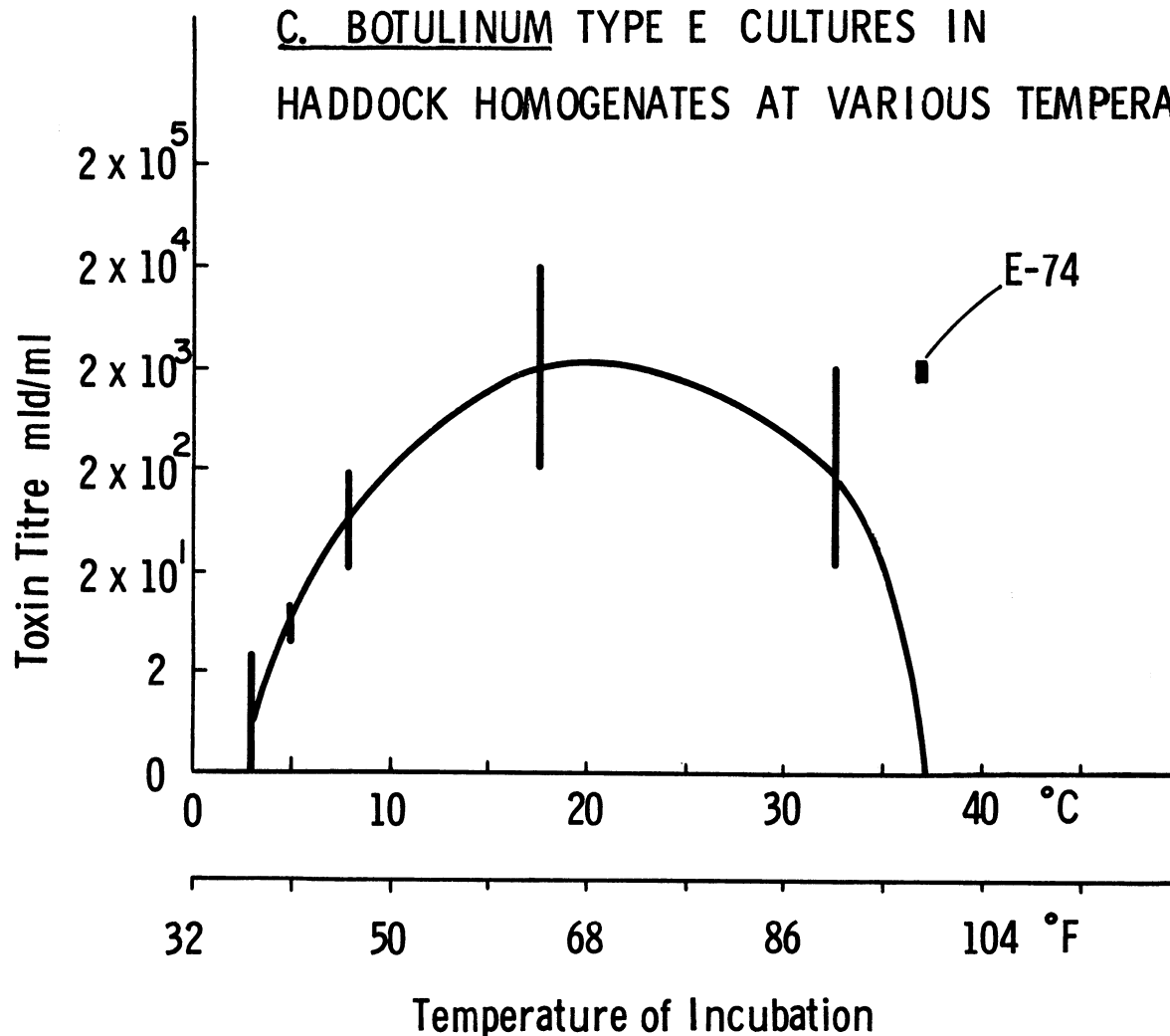
Fig. 3.   Toxin titres for 6 strains of C. botulinum Type E cultures
developed in haddock homogenates at various temperatures.

GROWTH OF <u>C. BOTULINUM</u> TYPE E IN TRYPTICASE PEPTONE
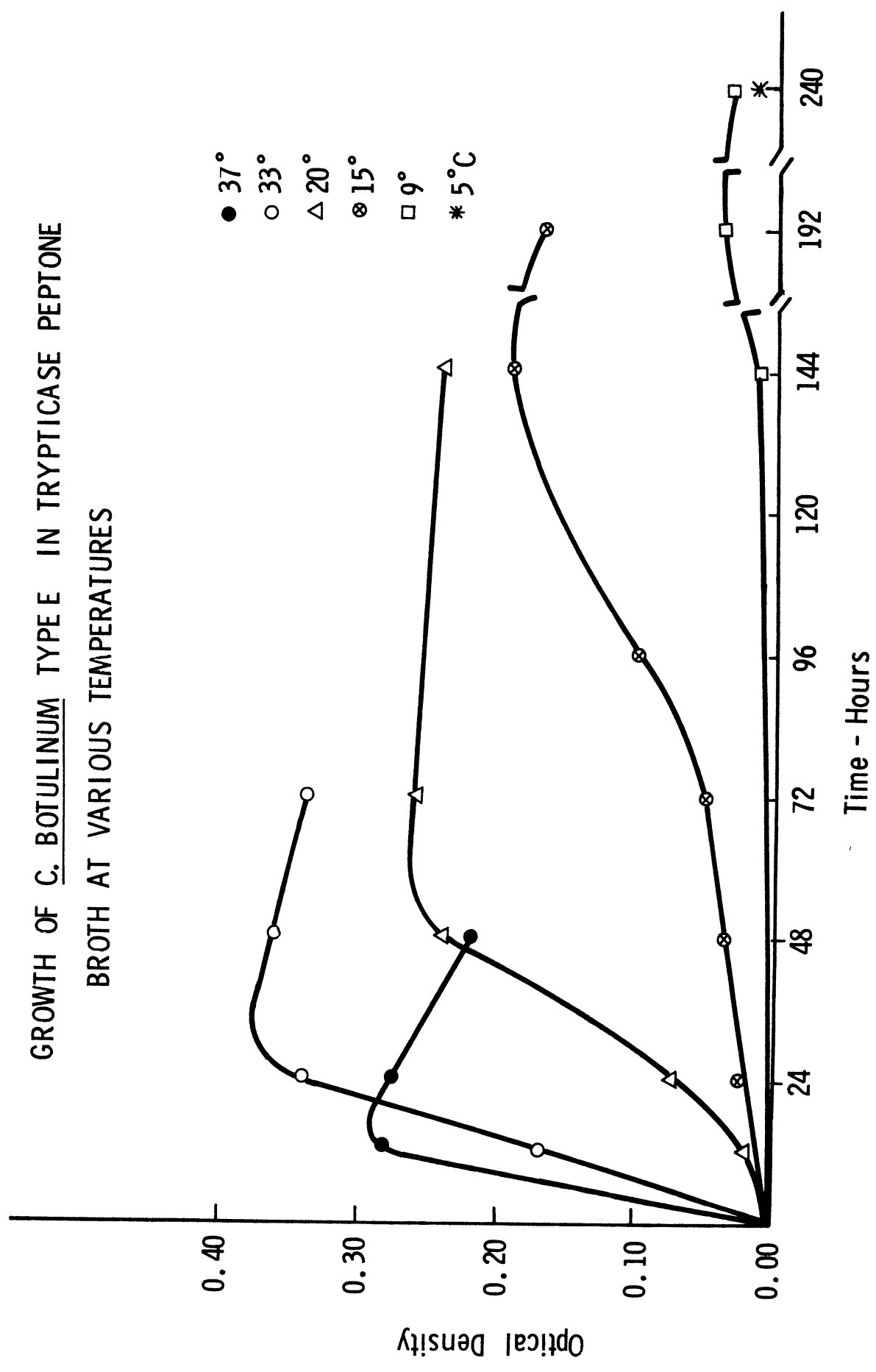BROTH AT VARIOUS TEMPERATURES

● 37°
○ 33°
△ 20°
⊗ 15°
□ 9°
＊ 5°C

Optical Density

Time - Hours

Fig. 4. Growth of <u>C. botulinum</u> Type E in trypticase peptone
broth at various temperatures.

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Effects of Media Variation on Toxin Production

Toxin production by C. botulinum Type E is being studied in various culture media. These include seafoods that are sterilized by radiation and heat, media with different nitrogen sources and sugars and media adjusted to various pH levels.

Seafood Substrates

Previously we observed that toxin production in clam juice was higher in heat sterilized media than in radiation sterilized juices. Since radiation sterilization of the media does not inactivate native enzymes, toxin production under these conditions may be variable, due possibly to toxin destruction by these enzymes.

A comparison was made between toxin production in haddock homogenates, which were not sterilized, and in homogenates which were sterilized by radiation and by heat. After sterilization the tubes of haddock homogenates were inoculated with approximately 10^6 spores per ml, incubated at 33°C, and then tested for toxin. Table III summarizes the results of this experiment. Significantly, no toxin was detected in the untreated sample, which was contaminated with other organisms.

TABLE III

PRODUCTION OF TOXIN IN HADDOCK HOMOGENATES INOCULATED WITH SPORES OF

C. botulinum TYPE E (IWANAI) AND INCUBATED AT 33°C FOR 48 HOURS

Sample Treatment	MLD Toxin
None	0
Heated	20-200
Irradiated	20-200
Irradiated and Heated	2000

In another experiment the toxin producing ability of the organism was tested in haddock homogenates at incubation temperatures of 33°C and in oysters after they had been heat or radiation sterilized. Tables IV and V summarize the results of these experiments. No toxin was detected in the irradiation sterilized oyster juice in tubes which had been inoculated with spores of three different strains of Type E. However, toxin was produced in the oyster

juice when heated after irradiation. A heat sensitive factor which is present in oysters but not in haddock homogenates has a deleterious effect on the preformed toxin.

TABLE IV

TOXIN PRODUCTION BY C. botulinum TYPE E IN HADDOCK HOMOGENATE

AND OYSTER JUICE STERILIZED WITH GAMMA RADIATION

AND INCUBATED AT 33°C FOR 5 DAYS

Strain	MLD Toxin	
	Haddock	Oysters
Vancouver Herring	>20	0
Seattle Forks	>20	0

TABLE V

TOXIN PRODUCTION BY C. botulinum TYPE E (IWANAI) IN HADDOCK HOMOGENATE

AND OYSTER JUICE INOCULATED WITH C. botulinum TYPE E

SPORES AND INCUBATED AT 20°C FOR 7 DAYS

Sample	MLD Toxin
Haddock Homogenate	
Irradiated and Heated	2000
Irradiated	2000-5000
Oysters	
Irradiated	0
Irradiated and Heated	20,000

Effect of Media on Toxin Production

 A comparison was made of the toxin producing ability of the six Type E strains used in our studies. The three media selected were beef infusion broth, trypticase peptone media and the media used by Duff in his comparison of toxin production by various Type E strains. The extent of toxin production in 200 ml of media was checked after incubation at 33°C for seven days. As can be seen from Table VI, the amount of toxin produced by the six strains in the three different media was not consistent. Wide variation in toxin titres can be expected, depending upon the strain of organism and medium used.

TABLE VI

TOXIN PRODUCTION IN THREE MEDIA BY $\underline{C}$. botulinum TYPE E

INCUBATED AT 33°C FOR 7 DAYS

| Strain | MLD/mℓ | | |
	Media D	TP	BI
Iwanai	$2\text{-}20 \times 10^2$	$2\text{-}20 \times 10^2$	200×10^2
Seattle Forks	$2\text{-}20 \times 10^2$	$20\text{-}200 \times 10^2$	$.2\text{-}2 \times 10^2$
E-74	$20\text{-}200 \times 10^2$	$20\text{-}200 \times 10^2$	20×10^2
Beluga	2×10^2	$2\text{-}20 \times 10^2$	2×10^2
Nanaimo	$20\text{-}200 \times 10^2$	$200\text{-}2000 \times 10^2$	---
Vancouver Herring	$2\text{-}20 \times 10^2$	200×10^2	20×10^2

D = Proteose Peptone - YE - 1% Dextrin
TP = Trypticase-Peptone - 1% Dextrose
BI = Beef infusion - 1% Dextrose

Effect of pH on Growth and Toxin Production

 The extent of toxin production by strain Iwanai in trypticase peptone media containing glucose was tested at pH 6.58, 6.9, 7.0 and 7.51. Growth after 72 hours of incubation did not occur at pH 4.98. As determined turbidimetrically, the greatest growth occurred at pH 7.8. In this narrow pH range no differences in toxin titres were found except at pH 7.51, where greater growth and gas production were observed (see Table VII).

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TABLE VII

TOXIN PRODUCTION BY C. botulinum TYPE E (IWANAI)
INCUBATED IN TRYPTICASE PEPTONE BROTH AT 33°C
FOR 72 HOURS AT VARIOUS pH LEVELS

| pH	MLD/Toxin
6.88	200-2000
6.90	200-2000
7.00	2000
7.51	2000-20,000

IRRADIATION STUDIES

The radiation resistance of the spores of the six Type E strains used
in our studies is being compared. The influence on radiation resistance of
such environmental factors as seafood homogenates, temperature and salt and
smoke extract is also being studied.

The description of the radiation source and dosimetric procedures used
in all of these studies is given in Appendix A.

Thoroughly washed spores are used in all of the work in order to min-
imize carry-over of medium constituents which may affect the radiation re-
sistance of the organisms. Prior to use, the spores are heated at 60-65°C
for 15 minutes. Following heat treatment, the organisms are diluted to the
desired concentration in the substrate being tested. Usually a concentra-
tion of 10^6-10^7 spores per ml is employed.

The spores for irradiation are contained in 5 ml sealed vials. These
vials are positioned in a rack which fits into a Dewar flask and has spaces
for 28 vials. The Dewar flask is positioned in the center well of the
cobalt-60 source. Dose rate measurements are determined in the same vials
in which the spores are irradiated. During dosimetry determinations the
vials are placed in the same position occupied by vials containing spores
suspension during irradiation. Figure 5 shows the dose rates in positions
occupied by the vials during irradiation. All irradiations are carried out
at melting ice temperatures (0°C).

After irradiation, the spores are diluted in sterile water and approp-
riate dilutions are sub-cultured into a counting medium. The medium pres-
ently employed for counting is fresh beef infusion agar.

N
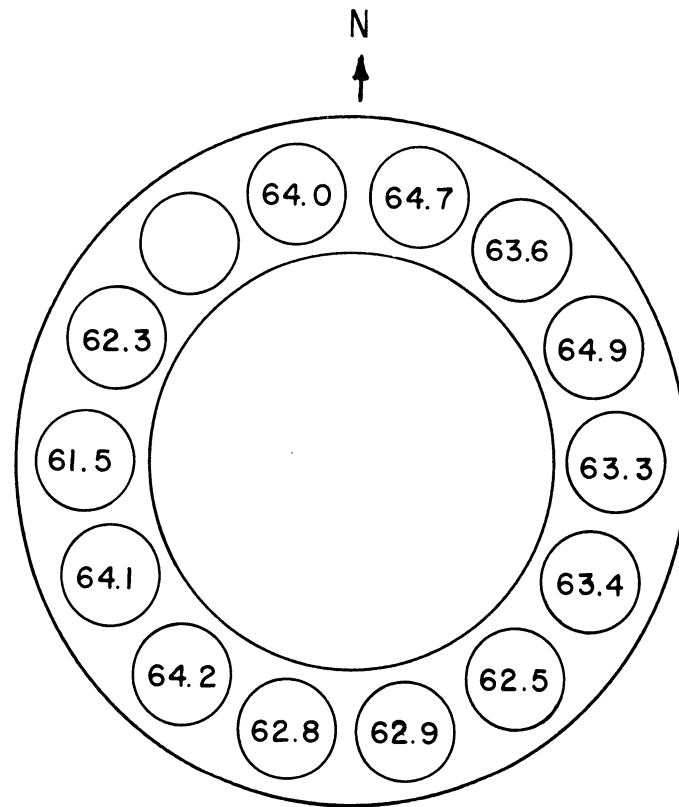
Top rack
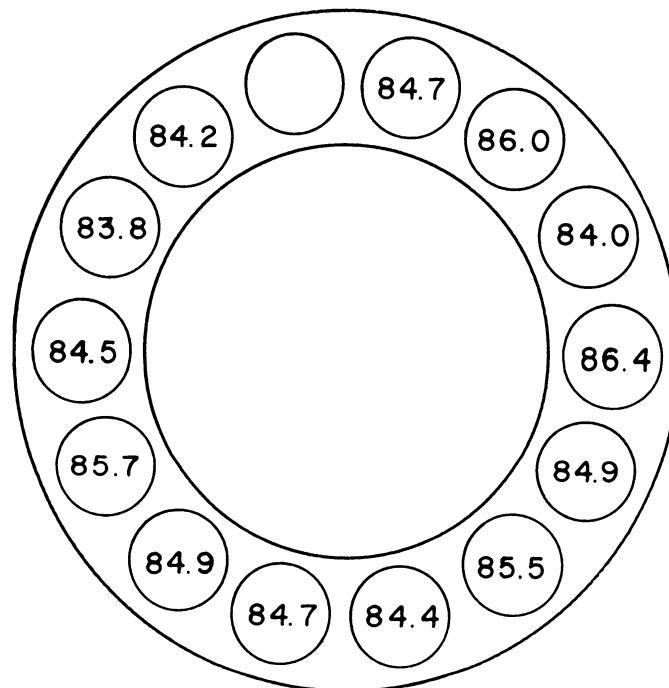
Bottom rack

Dose rate Kilorads / hour

Fig. 5. Gamma radiation dose rates in the position occupied by the spore suspensions during irradiation in the Phoenix Radiation Facility cobalt-60 source.

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The results of the irradiation of spores of the six Type E strains in M/15 phosphate buffer are presented in Figs. 6 and 7. The six strains show little difference in resistance to gamma radiation.

The results of irradiation of spores suspended in haddock homogenate and oyster juice is presented in Table VIII. The haddock homogenate and oyster juice offer appreciable protection to the spores during irradiation.

TABLE VIII

COMPARATIVE SENSITIVITY TO GAMMA RADIATION OF C. botulinum

TYPE E (SEATTLE FORKS) SPORES SUSPENDED IN PO4 BUFFER,

OYSTER JUICE, AND HADDOCK HOMOGENATE

Megarads	PO4 %	Log % Survivors in Oysters	Megarads	Log % Survivors in Haddock
0	100.0	100.0	0	100.0
.14	34.2	3.6	.11	14.8
.29	14.9	36.0	.22	14.3
.43	0.51	19.1	.34	17.5
.57	1.08	10.3	.45	12.3
.76	.046	5.91	.56	16.0
.86	.019	1.43	.67	8.1
1.1	.000021	0.236	.78	3.1

RESISTANCE OF Clostridium perfringens TYPE A SPORES TO IONIZING RADIATION*

Since very little work has been done on the radiation resistance of C. perfringens, it is of interest to compare the radiation resistance of these spores with that of C. botulinum spores.

Food poisoning has been known to result from eating foods in which cultures of C. perfringens Type A have inadvertently grown. Within the past few months, radiation sterilized bacon has been approved by the Federal Government as an acceptable food product. Other irradiated foods are being studied for similar clearance. These include foods treated with both irradiation sterilization and irradiation pasteurization processes. No data

*This section was written by T. F. Midura, J. T. Graikoski, L. L. Kempe, and N. A. Milone, of the Departments of Environmental Health and Chemical Engineering, The University of Michigan.

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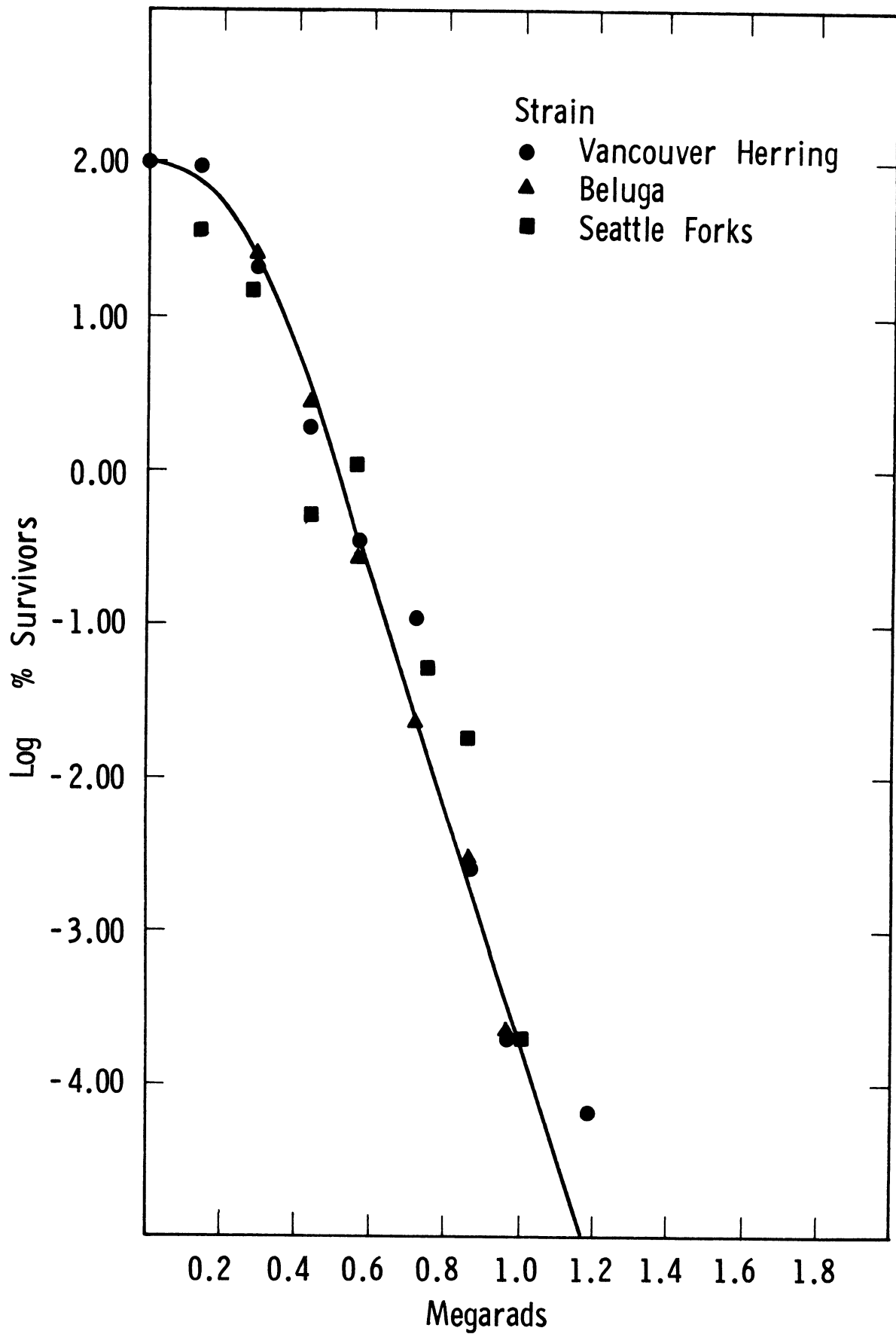
Fig. 6. Comparative resistance to gamma radiation of strains of C. botulinum Type E (Vancouver Herring, Beluga, Seattle Forks).
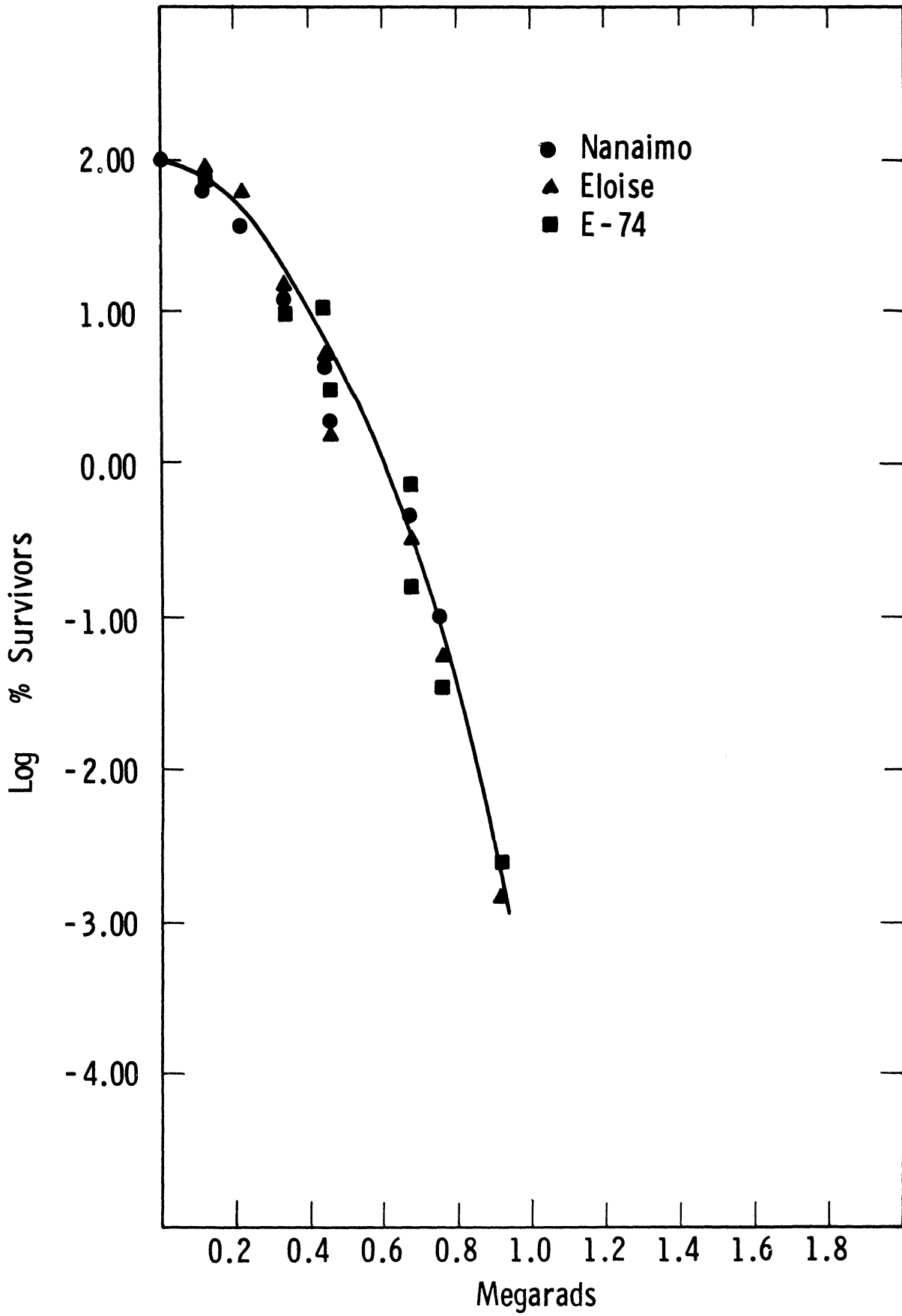
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Fig. 7. Comparative resistance to gamma radiation of strains of C. botulinum Type E (Nanaimo, Eloise, E-74).

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have been published regarding the radiation resistance of C. perfringens spores, which could conceivably be involved in determining the wholesomeness of irradiation processed foods. The present study is intended to apply to this void.

Spores of a Hobbs, "heat-resistant" strain were produced in a trypticase, soy broth medium. Purity of the culture was established by suitable identification criteria, such as: Gram reaction, anaerobiosis, lecitho-vitellin reaction, absence of motility, biochemical reactions, aerologic testing, and heat-resistance of the spores. In each instance, reactions were characteristically those of C. perfringens.

Spore suspensions were heat-shocked at 80°C for 10 minutes, which treatment also kills vegetative cells. Numbers of viable spores were determined by dilution counts using the Prickett tube technique and a modified Mossel's medium.

Approximately 10^6 spores per ml were suspended in M/15 phosphate buffer (pH 7.0). Quantities of 4 ml were dispensed into 5-ml ampules. The ampules were heat sealed and immersed in ice water, and the spore suspensions were irradiated with gamma rays from a cobalt-60 source at a rate of 74,000 rads per hour. A non-irradiated control sample was kept at 4°C. Surviving spores were counted as previously described.

As shown in Fig. 8 and Table IX, the D value of C. perfringens Type A spores in M/15 phosphate buffer was 0.23 megarads. Thus, C. perfringens spores appear to be more radiation resistant than C. botulinum Type E spores, which are stated[2] to have an approximate D value of 0.14 megarads.

This will be of significance in evaluating the survival of C. perfringens Type A spores in irradiated foods. It should be recognized that experience with other spores has indicated greater radiation resistance in foods than in phosphate buffer.[3]

TABLE IX

RADIATION RESISTANCE OF C. perfringens TYPE A SPORES,

SUSPENDED IN M/15 PHOSPHATE BUFFER AT pH 7.0,

TO GAMMA RADIATION FROM COBALT-60

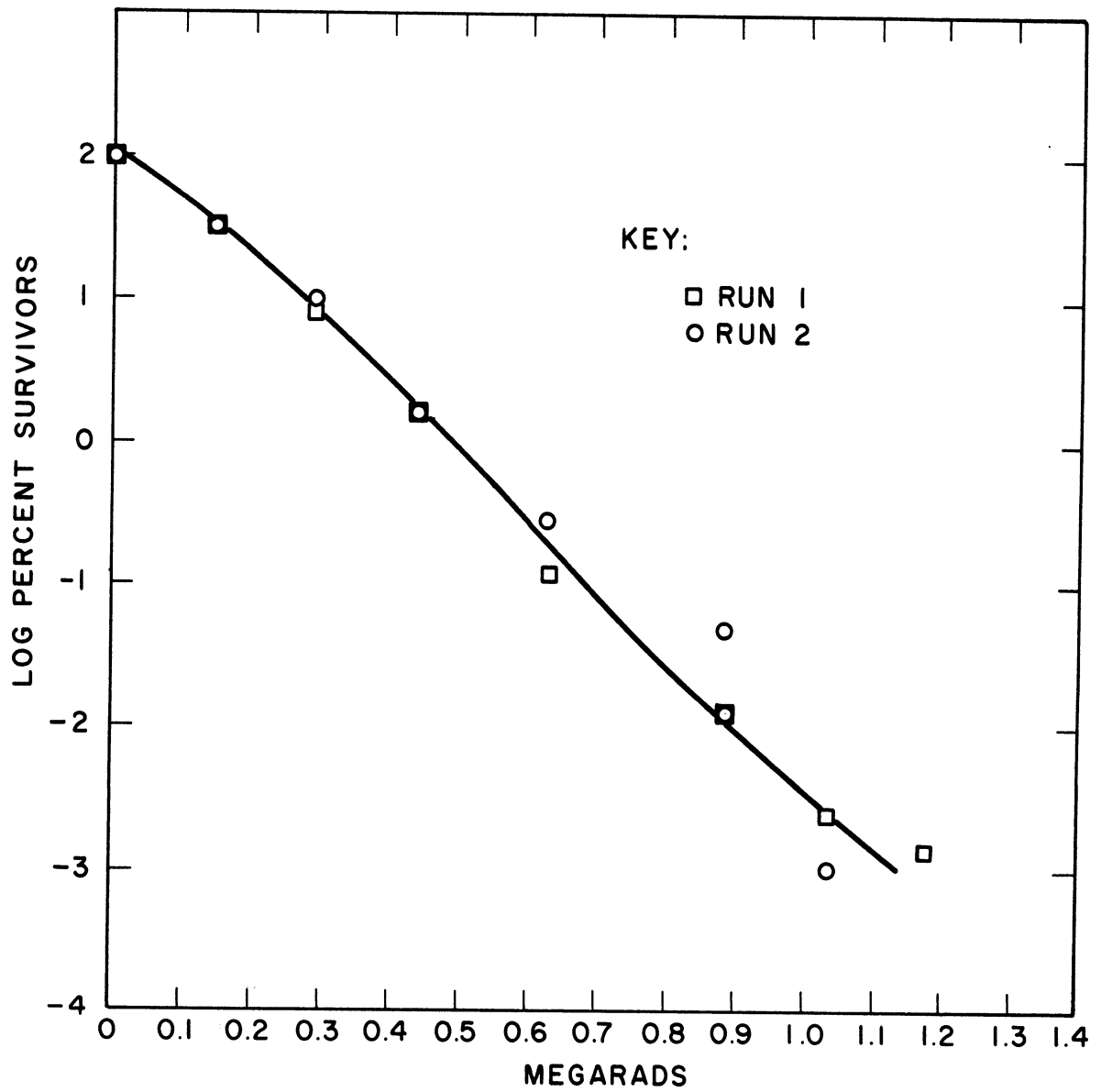| Megarad	Spores/ml	% Survivors	Log % Survivors
Run 1			
0.000	800,000	100.0	2.0
0.148	230,000	28.8	1.46
0.296	60,000	7.5	0.88
0.444	12,000	1.5	0.18
0.629	1,000	0.125	-0.90
0.888	100	0.0125	-1.90
1.036	20	0.0025	-2.60
1.184	10	0.00125	-2.90
Run 2			
0.000	1,000,000	100.0	2.0
0.148	300,000	30.0	1.48
0.296	110,000	11.0	1.04
0.444	17,000	1.7	0.23
0.629	3,000	0.3	-0.52
0.888	500	0.05	-1.30
1.036	10	0.001	-3.00

Fig. 8. Radiation resistance of C. perfringens Type A spores suspended in M/15 phosphate buffer to gamma radiation from cobalt-60.

CURRENT STUDIES

Studies in progress and to be started before Oct. 1, 1963 are as follows:

1. Long term studies at low incubation temperature are continuing. More cultures are to be assayed for toxin.

2. Some cultures which have incubated at the low temperatures for long periods of time will be incubated at higher temperatures to test for spore viability and subsequent toxin production.

3. Another low temperature experiment is being started. In this experiment additional food substrates, fresh fish another salt water species, smoked fish, both irradiated and heated, nutrient broths, will be compared. Emphasis will be placed on the effect of media variation on growth and toxin production at low temperatures.

4. Comparison of the radiation resistances of additional types of A, B and E strains is in progress. The effect of environmental conditions during irradiation is being studied.

5. Several media will be tested for their ability to stimulate germination and outgrowth of Type E spores.

6. The heat resistance of three Type E strains is being compared in order to correlate it with the irradiation resistance of these strains.

APPENDIX A

DOSIMETRY AND DOSE RATES IN AND AROUND THE COBALT-60 GAMMA RADIATION

SOURCES AT THE PHOENIX RADIATION FACILITY AND THE PHOENIX BUILDING

DESCRIPTION OF COBALT-60 RADIATION SOURCES

Two sources of cobalt-60 are used for research work under this project. The one used more often is located at the Phoenix Radiation Facility, where the microbiological laboratory is located. It is rated at 1600 curies as of March 1963. The other source is located at the Phoenix Memorial Laboratory. It is rated at 2700 curies as of March 1963.

The Phoenix Radiation Facility (PRF) source consists of 42 cobalt-60 aluminum jacketed rods arranged in a cylindrical holder 8-3/8 in. (I.D.) and 10-5/8 in. (O.D.). There are 43 equivalent concentric positions for the rods in this holder, with one vacant position in the northwest direction. The cylindrical holder is contained in a stainless steel capsule. The capsule was evacuated and the air replaced with helium after the introduction of the cobalt-60 rods. This arrangement was necessary since the immersion of cobalt-60 aluminum jacketed rods in water caused some corrosion problems.

The source capsule is situated on an elevator mechanism in the source room. The room is 11 ft long and 8 ft wide, with 4 ft walls of concrete and a 2 ft concrete ceiling.

The radiation source is lowered in a 16 ft well (into about 12 ft of water) located in the center of the source room when entrance is necessary for placement of the samples to be irradiated. When raised, the cobalt-60 source enters into a perforated stainless steel cage which acts as a shield against accidental tipping of the source from the elevator. Figure A-1 shows the stainless cage located in the source room.

The other source, located at the Phoenix Memorial Laboratory, has a similar arrangement except that the 35 cobalt-60 rods are arranged more closely, in a smaller circle. This arrangement combined with a larger quantity of cobalt-60 provides a source with higher intensity. But the source is not as convenient for large samples since the sample size is limited to 7.5 cm (O.D.) as compared with 18.2 cm (O.D.) at the Phoenix Radiation Facility.

DOSIMETRY PROCEDURES

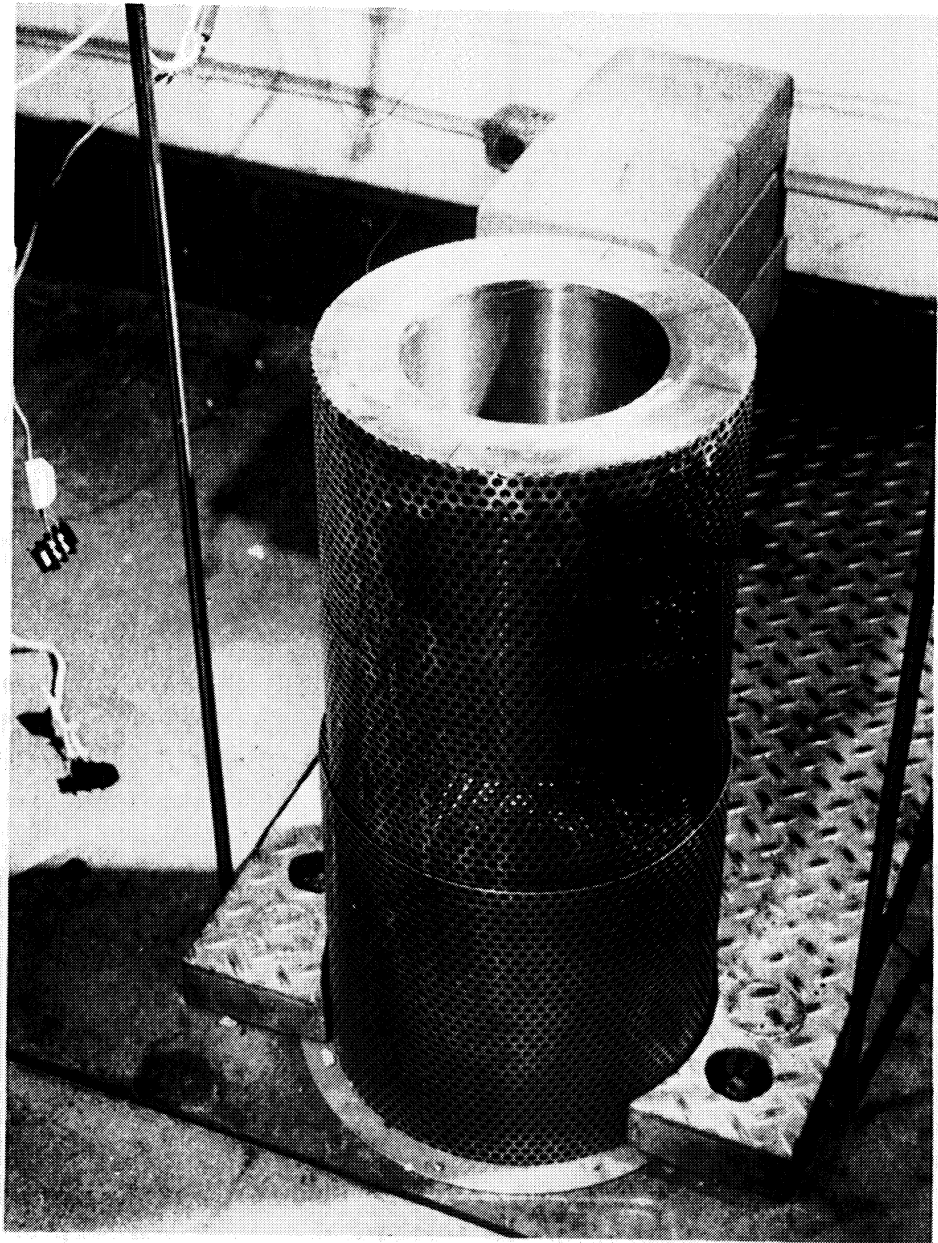A calibration curve was made to determine the extinction coefficient

Fig. A-1. Stainless steel cage for Phoenix Radiation Facility cobalt-60 source.

of solutions containing ferric ions.

The dose rates were determined by measuring the amount of oxidation of ferrous ions in a solution of ferrous ammonium sulfate as described in the ASTM method.[4]

Calibration Curve

Procedure.—A solution of ferric sulfate of approximately 1/10 molarity was made up in 0.8N sulfuric acid. This solution was standardized by reduction of Fe^{3+} to Fe^{2+} in a Jones reductor and subsequent oxidation of Fe^{2+} by standardized permanganate solution.

A number of solutions containing varying amounts of Fe^{3+} were made up from the above solution by dilution. The optical densities of these solutions were determined on a Beckman DU Spectrophotometer at a wavelength of 305 mµ and a slit width of 0.5 mm, and using quartz cells. 0.8N sulfuric acid solution was used as a blank for the spectrophotometer determinations. All determinations were made at a constant temperature of 22°C.

The gamma dose equivalent of the concentration of ferric ions in the solutions was calculated as outlined in the ASTM method,[4] the only change being that of using a G value of 15.6.

Results.—The results obtained for optical densities of the solutions containing varying amounts of ferric ions are noted in Fig. A-2 and Table A-I. The conversion of ferric ion concentration to absorbed dose in rads is shown in Fig. A-3.

The average extinction coefficient as obtained by the method of least squares applied to the results was found to be 2140.6 at 22°C. (Using a temperature coefficient of 0.7% per degree centigrade, the value obtained for extinction coefficient at 25°C is 2185.5.)

Recently, there has been some interest shown in the measurement of optical density of Fe^{3+} ion containing solution at wavelengths other than the normally used 304 or 305 mµ. It has been claimed that the extinction coefficient and hence the sensitivity is much higher at 224 mµ.[5] Another claim indicates that at 275 mµ,[6] even though the extinction coefficient is lower, there is the advantage of no temperature dependence.

Some work was conducted in this laboratory along this line, and the results are shown in Fig. A-4. At 22°C, the extinction coefficients at wavelengths of 224 mµ and at 275 mµ are estimated to be 5230 and 1790 respectively.
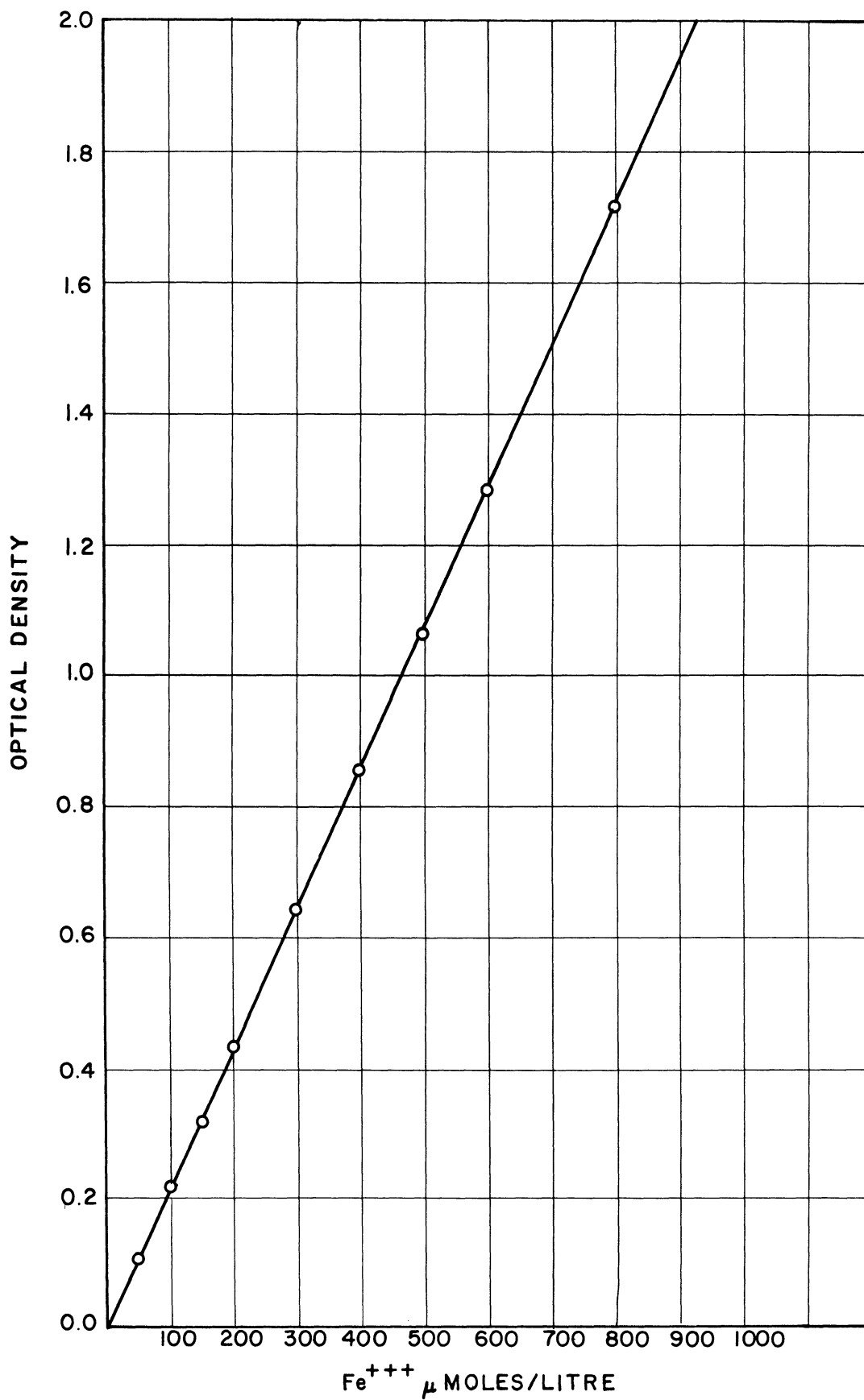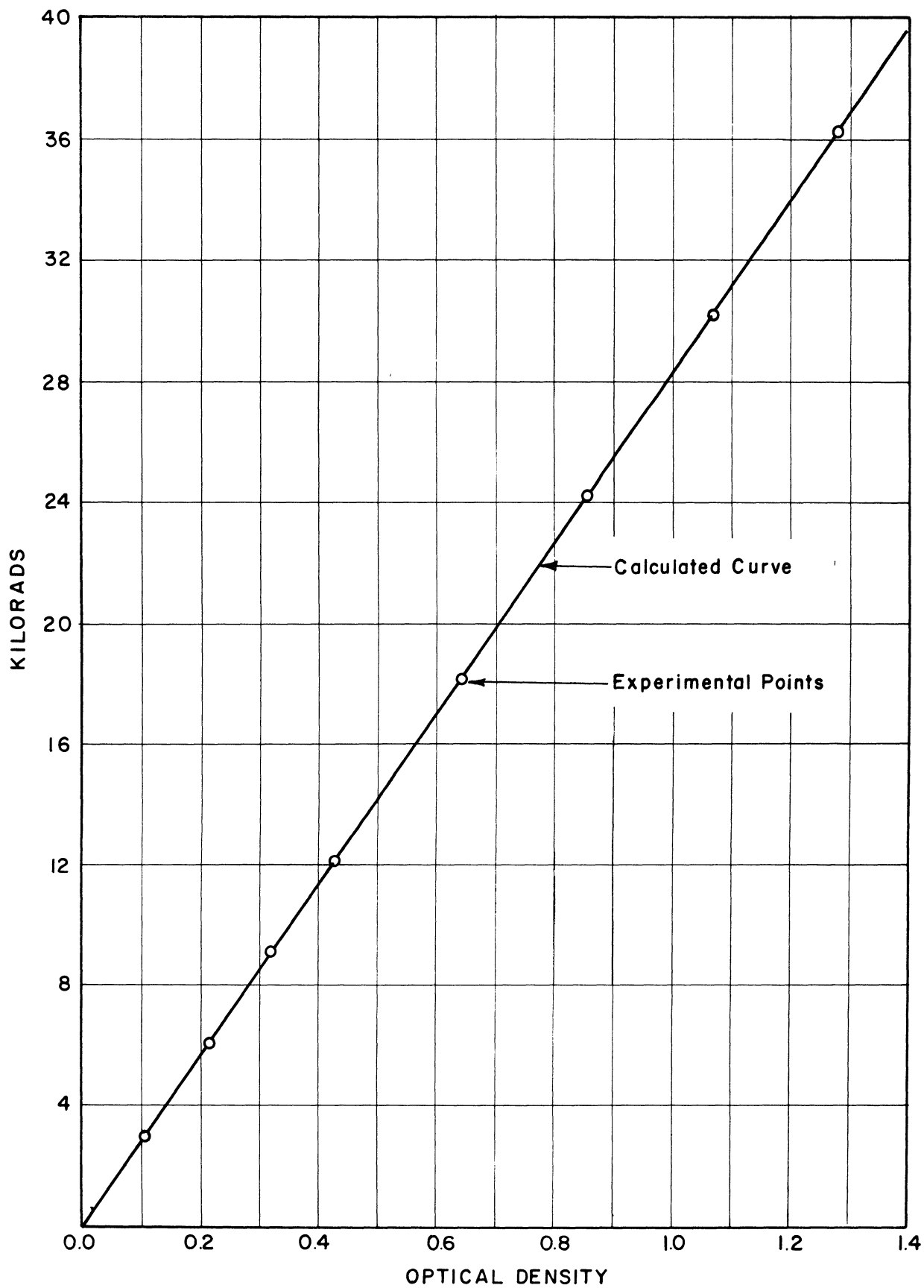
Fig. A-2. Optical density vs. concentration of Fe^{+3} in 0.8 NH_2SO_4.

Fig. A-3. Calculated gamma irradiation dose vs. optical density of Fe^{+3} solutions in 0.8 NH_2SO_4.
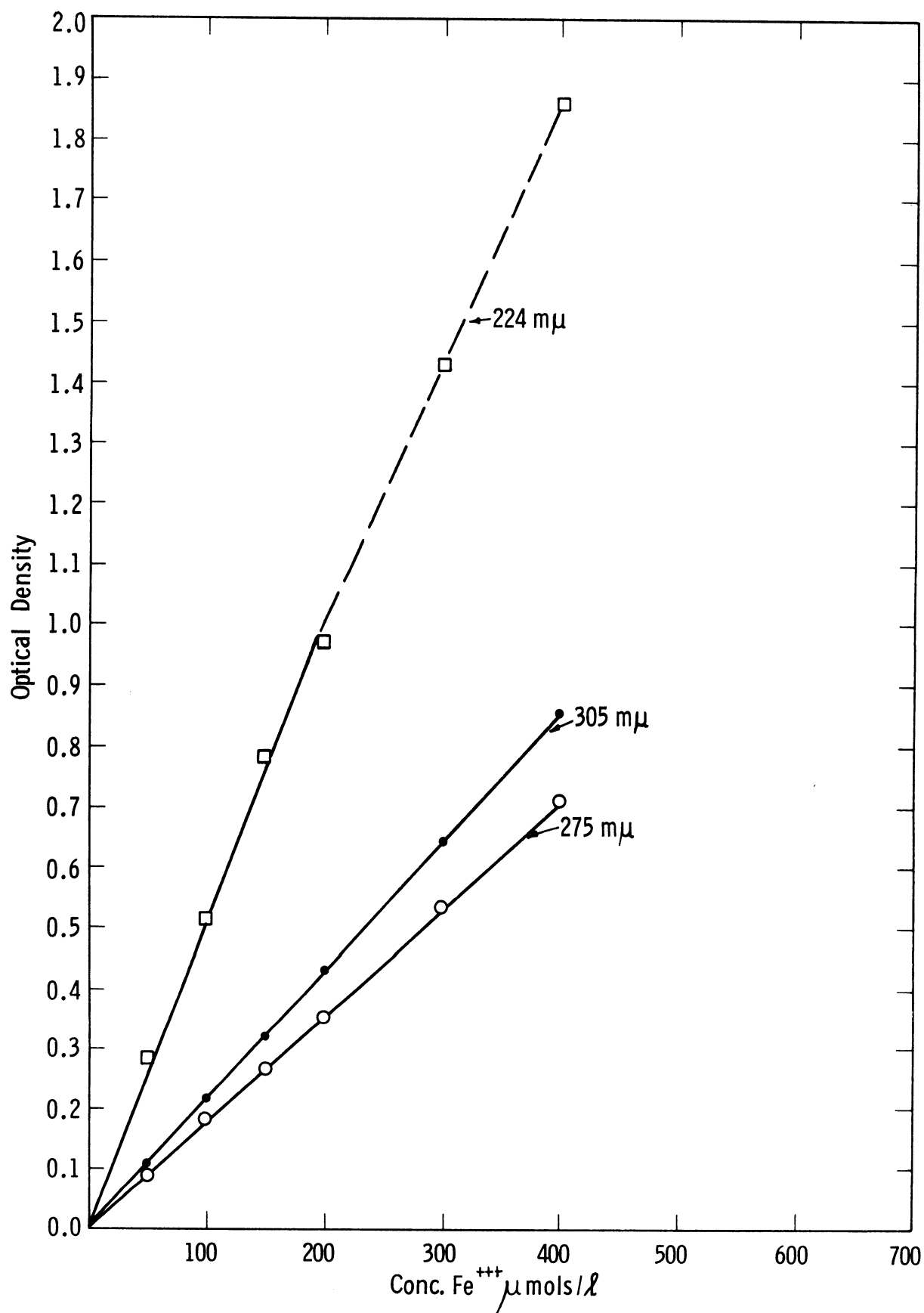
Fig. A-4. Optical density vs. concentration of Fe^{+3} solution at 22°C and various wavelengths.

TABLE A-I

OPTICAL DENSITY VS. CONCENTRATION OF Fe^{3+} SOLUTION

Concentration (mols/ℓ)	Temp. (°C)	Optical Density Observed*	Optical Density Corrected**	Ext. Coeff. a = A/C[+]	Equivalent Dose kilorads[++]
99.8	22	.213	.215	2154.3	6.0
149.8	22	.319	.317	2116.1	9.1
199.6	22	.431	.428	2144.3	12.1
299.5	22	.640	.642	2143.6	18.1
399.5	22	.858	.856	2142.7	24.2
499.1	22	1.065	1.063	2129.8	30.2
599.1	22	1.285	1.287	2148.2	36.2
799	22	1.713	1.710	2140.2	48.3

*Average extinction coefficient by the method of least squares = 2140.5 at 22°C.
**Optical density is corrected for cell deviations.
[+]Ext. coeff. = A/C optical density/mols/ℓ since path length = 1 cm.
[++]Equivalent gamma dose = concentration x 944/G as per ASTM method, where G = 15.6.

Gamma Dose Evaluation

The above results could be used for gamma dose evaluation as follows:

After determining the optical density of a solution of ferrous ammonium sulfate which has been irradiated for a measured amount of time, the dose provided by the radiation field could be determined by the use of Fig. A-3, which relates the amount of ferric ions formed with the total dose.

Alternatively, the dose could be calculated as follows:

$$\text{Dose (kilorads)} = \frac{\mu \text{ mols Fe}^{3+}}{\text{liter}} \times \frac{944}{G} \times 10^{-3}$$

$$\text{Concentration} \quad \frac{\mu \text{ mols Fe}^{3+}}{\text{liter}} = \frac{\text{optical density}}{\text{ext. coefficient*}} \times 10^6$$

---

*The extinction coefficient is defined here as the optical density of a solution of concentration 1 mol/ℓ through a path length of 1 cm.

An elaboration of these equations can be found in the ASTM method.[3]

Combining these two equations and using G = 15.6 and ext. coeff. = 2140.6 at 22°C, one obtains

$$\text{Dose (kilorads)} = \text{optical density} \times \frac{1}{\text{ext. coeff.}} \times 10^6 \times \frac{944}{G} \times 10^{-3}$$

$$= \text{O.D.} \times \frac{1}{2140.6} \times 10^6 \times \frac{944}{15.6} \times 10^{-3}$$

$$= \text{O.D.} \times 28.3 \text{ at } 22°C.$$

The number 28.3 will be noted as the dose factor for further reference. It is developed for use with the spectrophotometer at the Phoenix Radiation Facility and is valid only at 22°C. The dose rate provided by the radiation field can be obtained by dividing the total dose by the time of irradiation. The corrections to be applied for measurements at temperatures other than 22°C are given in the following section.

Temperature Correction

Since the extinction coefficient has the rather large temperature co-efficient of +0.7 percent per degree centigrade, a correction should be made if the optical density measurements are determined at a temperature other than the temperature at which the calibration curve was determined.

$$\text{Dose (corrected)} = \frac{\text{dose measured at } T_2}{1 + .007(T_2 - T_1)}$$

where

T_1 = temperature at which calibration curve was made,

T_2 = temperature at which optical density of unknown sample is read.

Table A-II lists some values of correction factors and dose factors for use in measurements made at the Phoenix Radiation Facility, where the calibration curve was made at 22°C.

TABLE A-II

CORRECTION FACTORS AND DOSE FACTORS FOR USE IN MEASUREMENTS
MADE AT THE PHOENIX RADIATION FACILITY

Temp. °C of Measurement of O.D.	Correction Factor	Dose Factor
20	1.0014	28.7
21	1.007	28.5
22	1.00	28.3
23	.993	28.1
24	.986	27.9
25	.979	27.7
26	.972	27.5
27	.966	27.3

Source Calibration

The dose rates provided by the cobalt-60 gamma source at the Phoenix Radiation Facility and the Phoenix Memorial Laboratory were measured by using the ferrous sulfate dosimeter.

Solutions of ferrous ammonium sulfate in 0.8N sulfuric acid were placed in the radiation field in screw capped glass vials. The vials were approximately 5 cm high and 10 mm O.D. For dosimetry in the center well, styrofoam slugs were cut out to fit snugly in the well, and the vials were placed in holes bored out to provide axial variation of distance. These slugs were 2 in. thick and six of them were used since the center well is 12 in. deep. They were placed on top of one another to provide measurements on various planes. For dosimetry outside of the center well, the vials were placed on blocks of wood, so that the volume of the solution was centered on the mid-plane of the gamma source and at measured distances from the outside edge of the source cage.

The vials for measurements at the Phoenix Radiation Facility were irradiated for amounts of time ranging from 10 minutes to 2-1/2 hours, depending upon various limitations. After equilibration of the solution vials to the instrument room temperature, the optical densities of the solutions were measured to evaluate the dose.

The radiation field at the cobalt-60 source at the Phoenix Memorial Laboratory was mapped in the same manner, except that the time of irradiation was much smaller.

Raising and lowering the cobalt-60 source in the cave at the Phoenix Memorial Laboratory takes a considerable amount of time. The time of irradiation of the dosimetry samples was measured from the moment the "source up" light came on. Since the samples for dosimetry in the center well were irradiated for two minutes, additional radiation was received by the samples while the source was being raised and lowered into position. This introduced a significant error which had to be corrected for. The correction factor was established by raising the source until the light came on, immediately lowering the source, and then measuring the dose received by a separate batch of samples during this period of time.

Another method of obtaining the correction factor would be to get dose evaluation at the point of investigation for various lengths of time. If the dose is then plotted against time, the straight line would not pass through the origin but would rather have an intercept equal to the additional dose of radiation received. The slope of the line would be the correct dose rate.

The results are correlated in Tables A-III through A-VII and Figs. A-5 through A-11. Some measurements were carried out in duplicate, and the values reported on tables are averaged. Even though the measurements of dose rates were carried out over a period of time, all data are adjusted for decay of cobalt-60 to show dose rates as of January 1, 1963.

TABLE A-III

DOSE RATES IN THE CENTER WELL OF THE

PHOENIX RADIATION FACILITY COBALT-60 SOURCE

(January 1, 1963)

Axial Distance from Bottom of Center Well (cm)	Dose Rates at Positions*				
	North		Centerline	South	
	1	2	3	4	5
2	81.4	71.9	71.3	74.4	77.1
12	101.6	94.9	87.2	90.4	99.7
22			64.7	66.5	69.6

	West		Centerline	East	
	6	7	8	9	10
7	96.5	86.7	85.1	88.7	96.4
17	93.0	83.0	80.4	84.0	91.1

*See Fig. 5 for positions.

TABLE A-IV

PHOENIX RADIATION FACILITY COBALT-60 SOURCE AXIAL DOSE RATES*

(January 1, 1963)

Distance from Bottom of Center Well (cm)	Dose Rate at Centerline	3.8 cm from Centerline (KRads/hr)**	7.6 cm from Centerline (KRads/hr)**
2	71.3	75.6	79.3
7	85.1	87.7	96.5
12	87.2	92.7	101.2
17	80.4	84.0	92.1
22	64.7	68.1	72.1
27	46.1	45.0	40.7

*All dose rates are averaged assuming concentric symmetry.
**The dose rates at 3.8 and 7.6 cm are the average of two values obtained at east and west positions from the centerline.

TABLE A-V

DOSE RATES AROUND THE PHOENIX RADIATION FACILITY

COBALT-60 SOURCE ON MIDPLANE

(January 1, 1963)

Distance from Outer Face of Basket (cm towards north)	Distance from Centerline of Source (cm)	Dose Rate (KRads/hr)
∼0.5	15.5	73.3
3	18	49.4
10.2	25.2	25.6
17	32	15.6
19	34	14.8
25	40	9.32
40	55	4.63
50	65	3.10
65	80	2.03
80	95	1.43
100	115	.960
125	140	.655
150	165	.469

TABLE A-VI

AXIAL DOSE RATES IN THE CENTER WELL OF

PHOENIX MEMORIAL LABORATORY COBALT-60 SOURCE

(January 1, 1963)

Axial Distance from Bottom of Center Well (cm)	Corrected* Dose Rate at Positions** (KRads/hr)		
	1	2 (Centerline)	3
2	270	267	273
7	341	326	339
12	347	333	347
17	342	331	342
22	307	286	290
27	196	196	196

*See text for details about correction.
**See Fig. 9 for explanation of positions.

TABLE A-VII

DOSE RATES AROUND THE PHOENIX MEMORIAL LABORATORY

COTABLT-60 SOURCE ON MIDPLANE

(January 1, 1963)

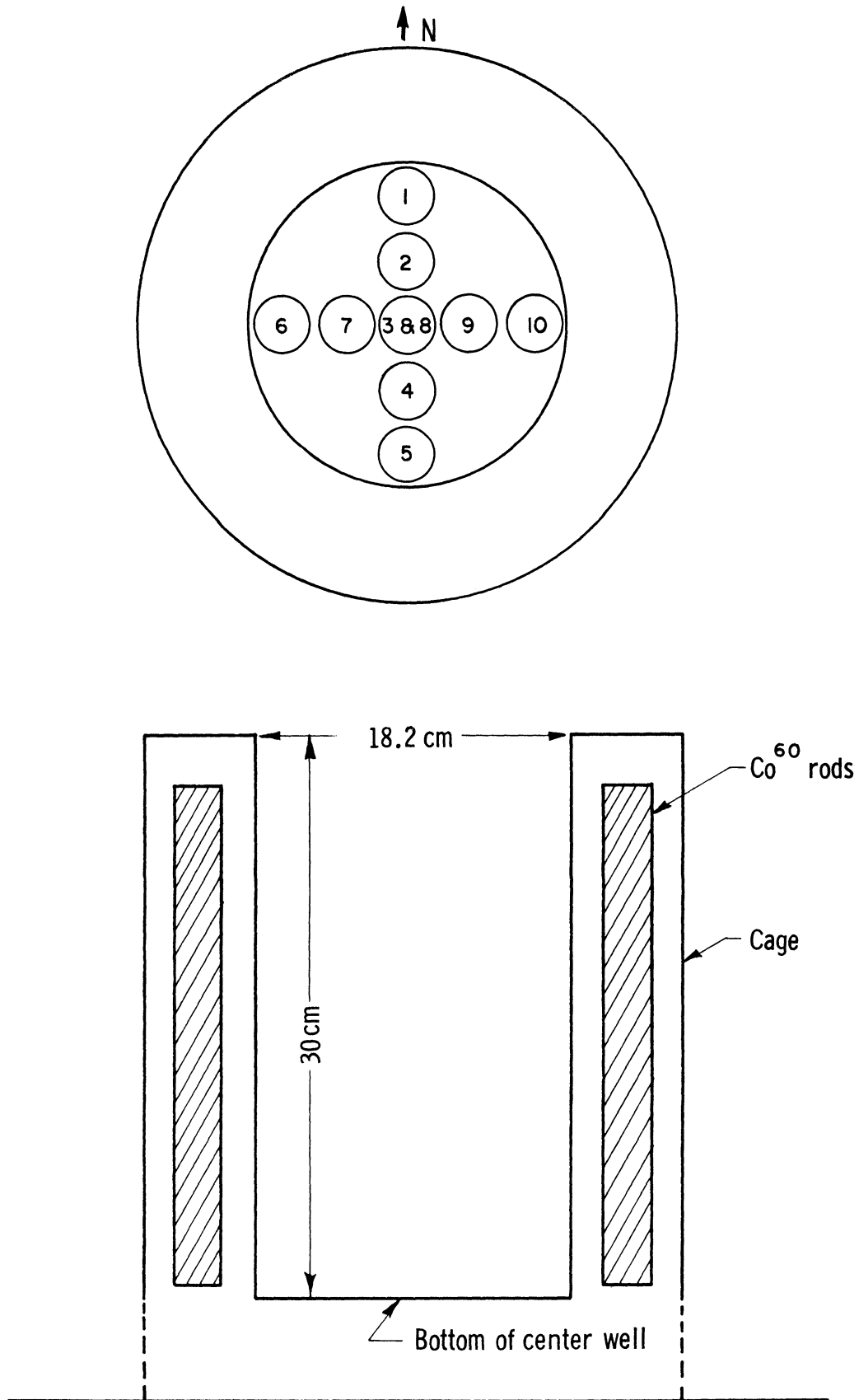| Measured Distance From Outside of Cage (cm)	Distance from Centerline (cm)	Dose Rate (KRads/hr)
~.5 (edge of cage)	9.5	164
5	14	95.2
10	19	53.7
15	24	35.0
20	29	25.7
30	39	14.7
40	49	9.71
50	59	6.47
60	69	4.52
75	84	3.32
90	99	2.26

Fig. A-5. Vial positions for irradiation in center well of
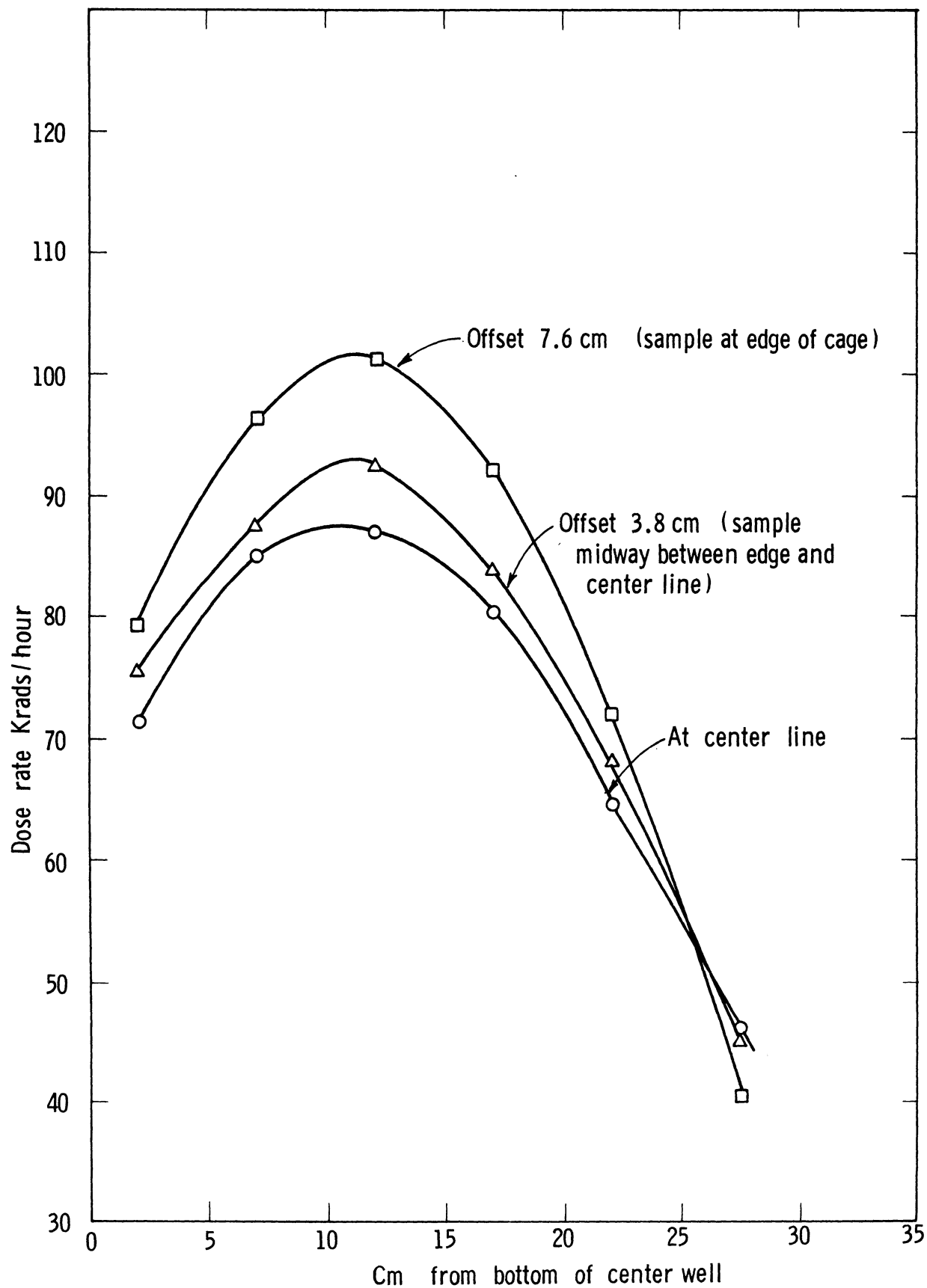Phoenix Radiation Facility cobalt-60 source.

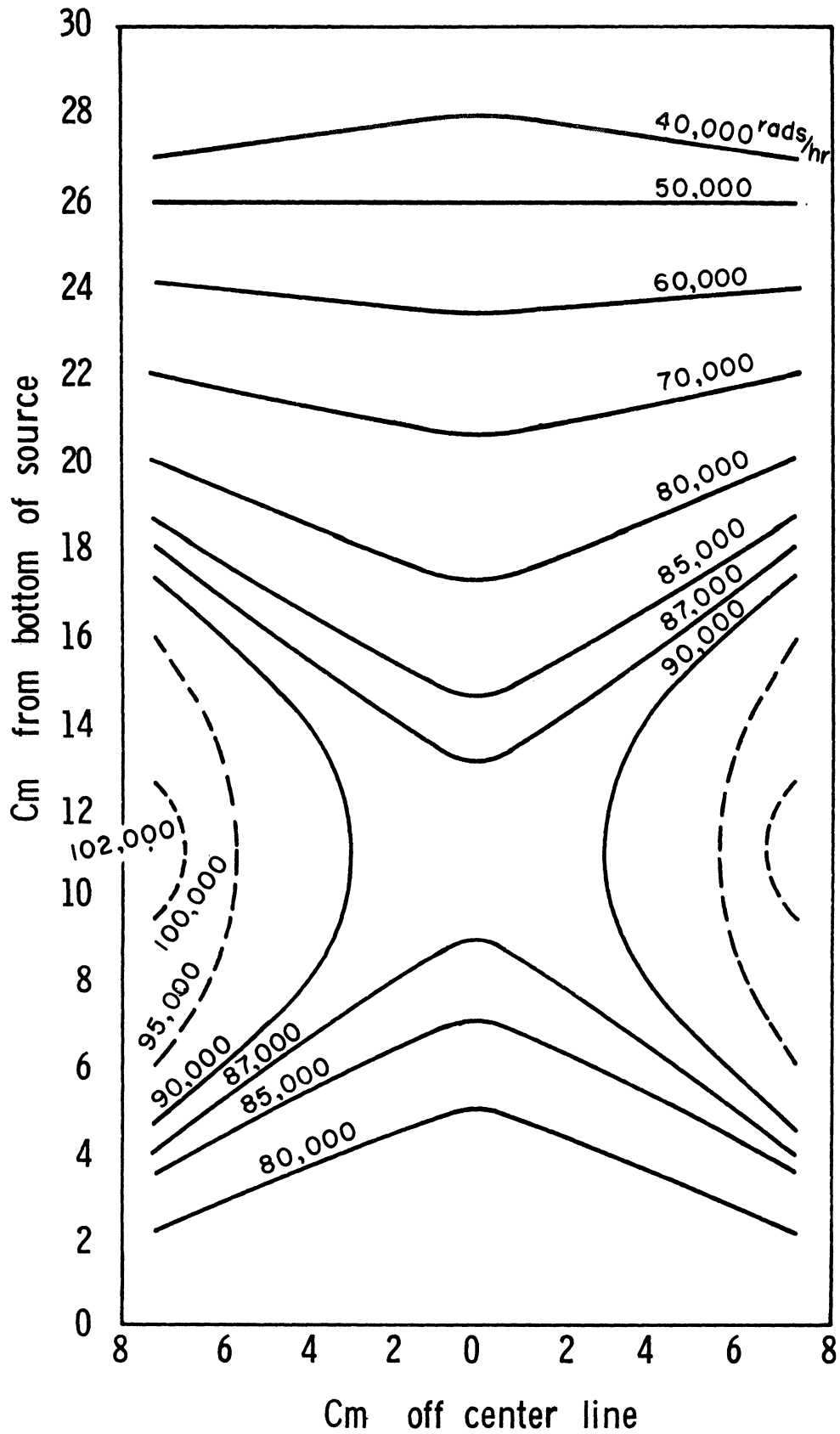Fig. A-6. Axial dose rates, Phoenix Radiation Facility cobalt-60 source (January 1, 1963).

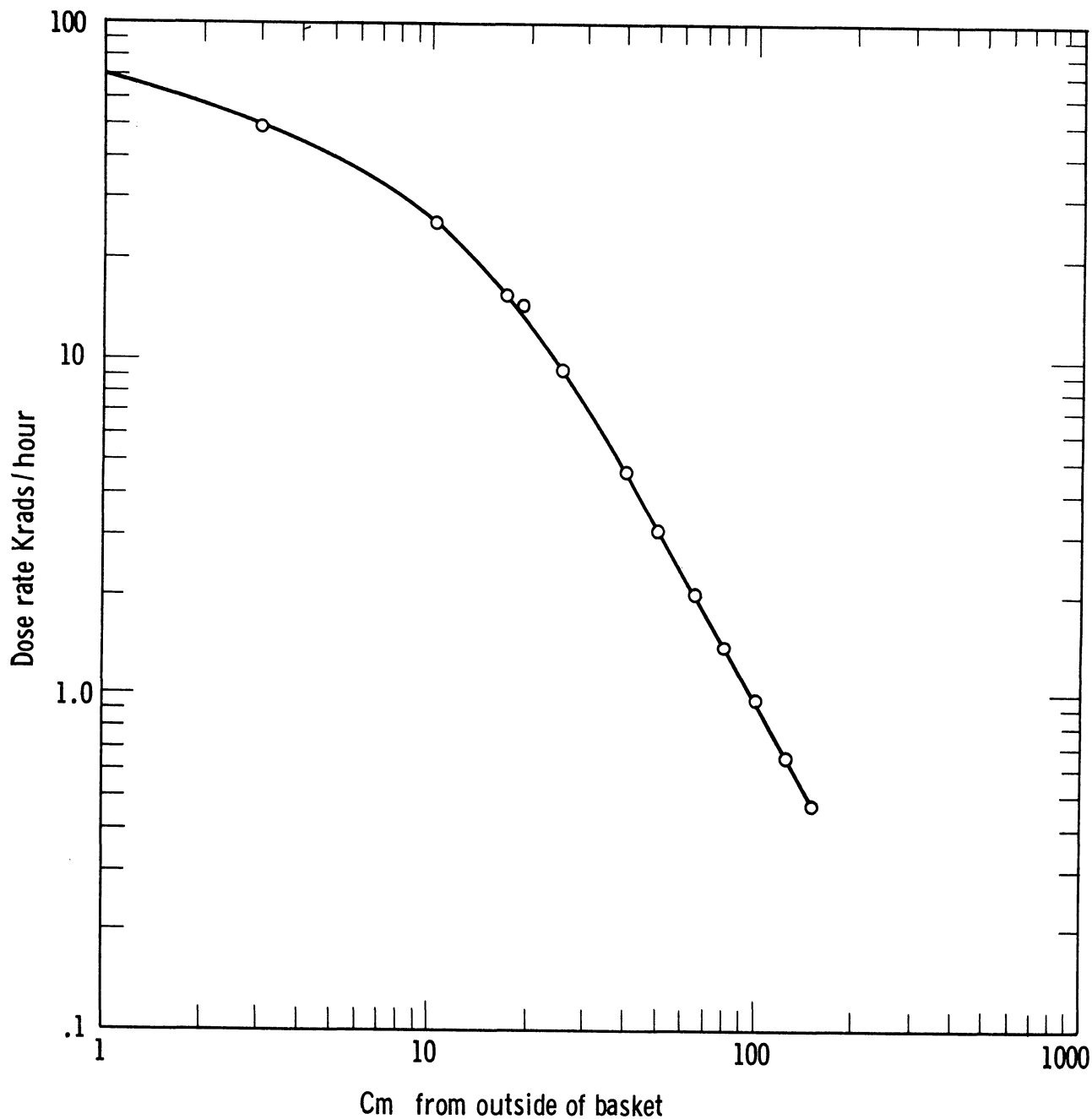Fig. A-7. Isodose curves, Phoenix Radiation Facility cobalt-60 source (January 1, 1963).

Fig. A-8. Dose rates on midplane and outside of cage, Phoenix
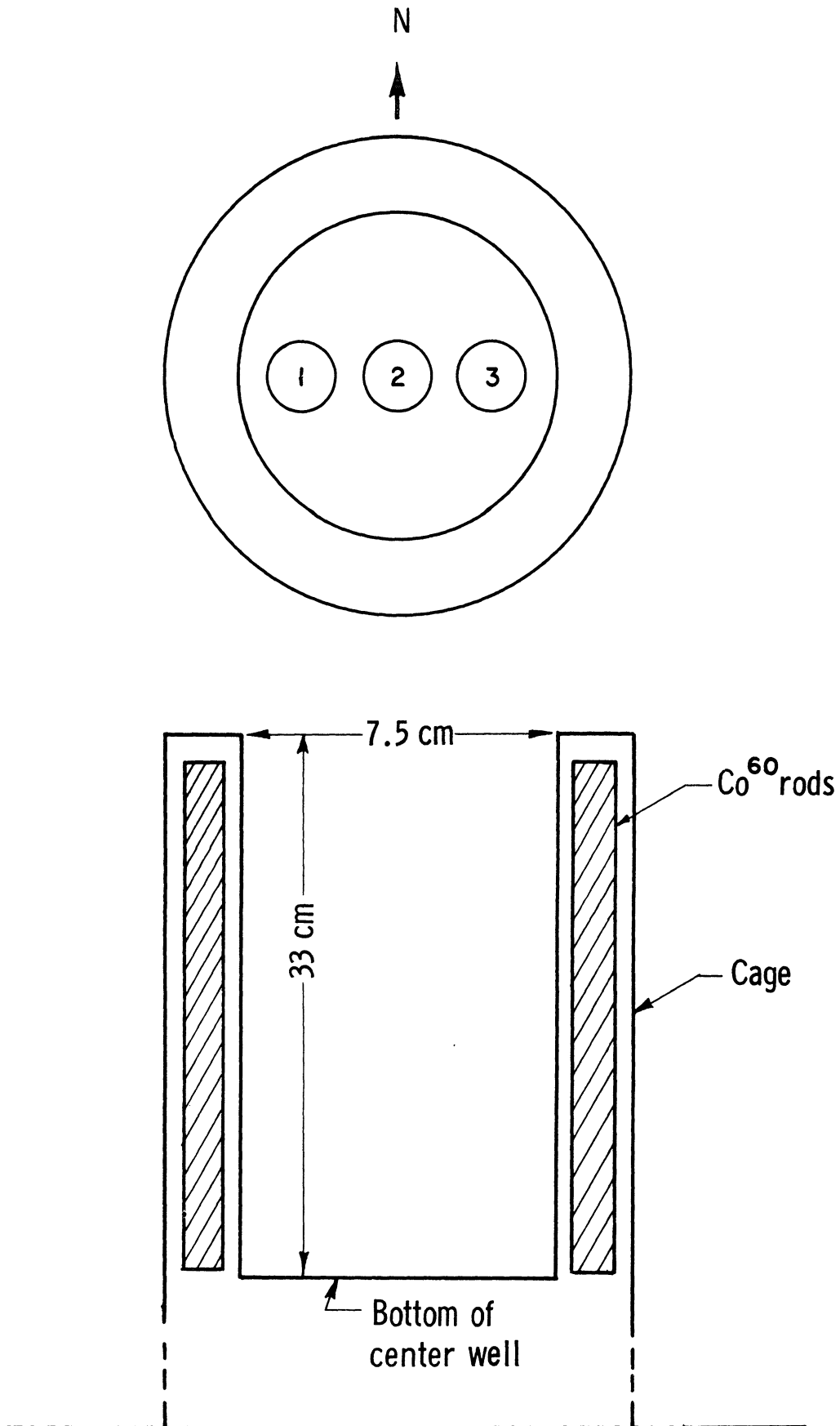Radiation Facility cobalt-60 source (January 1, 1963).

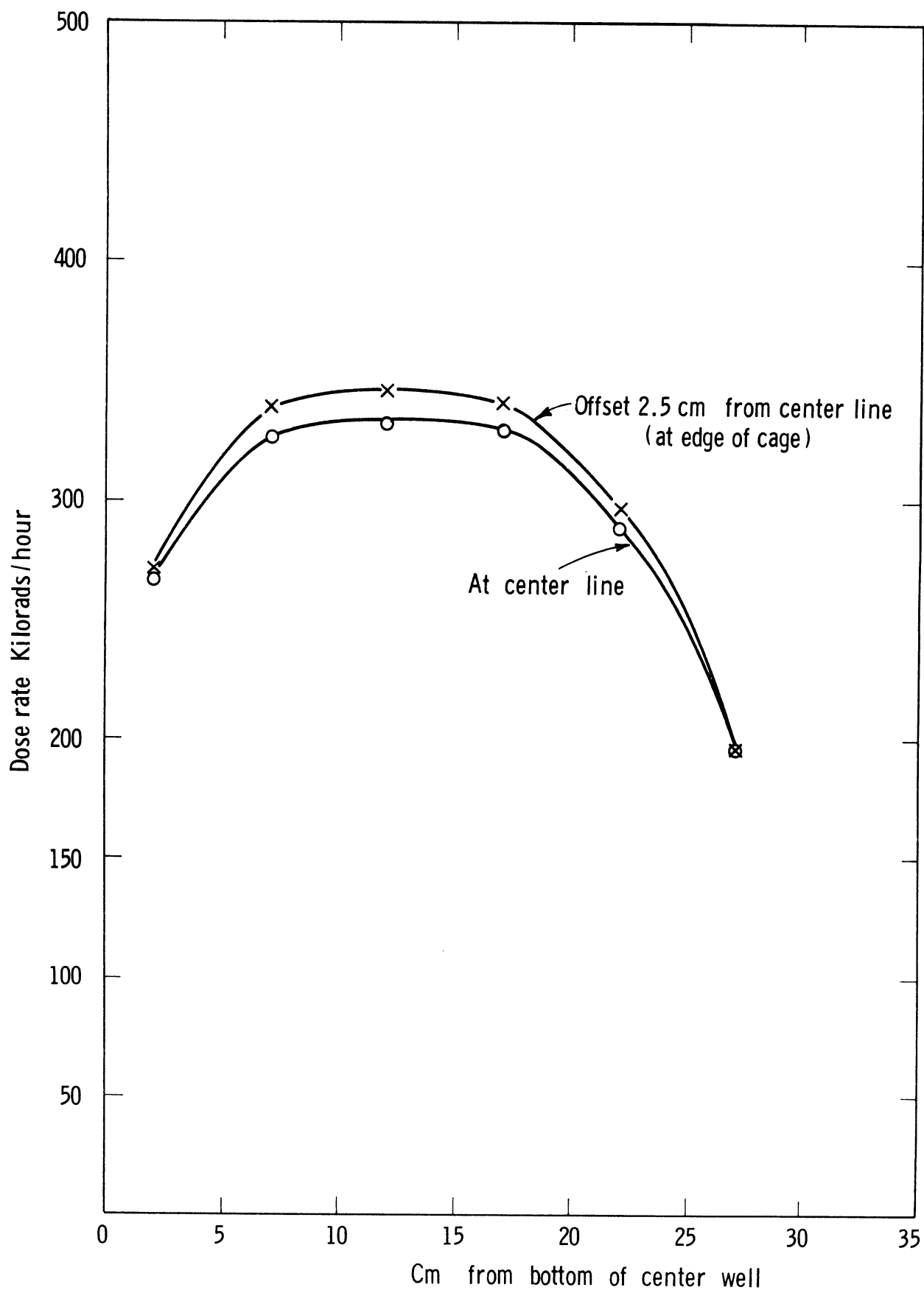Fig. A-9. Vial positions for irradiation in center well of Phoenix Memorial Laboratory cobalt-60 source.

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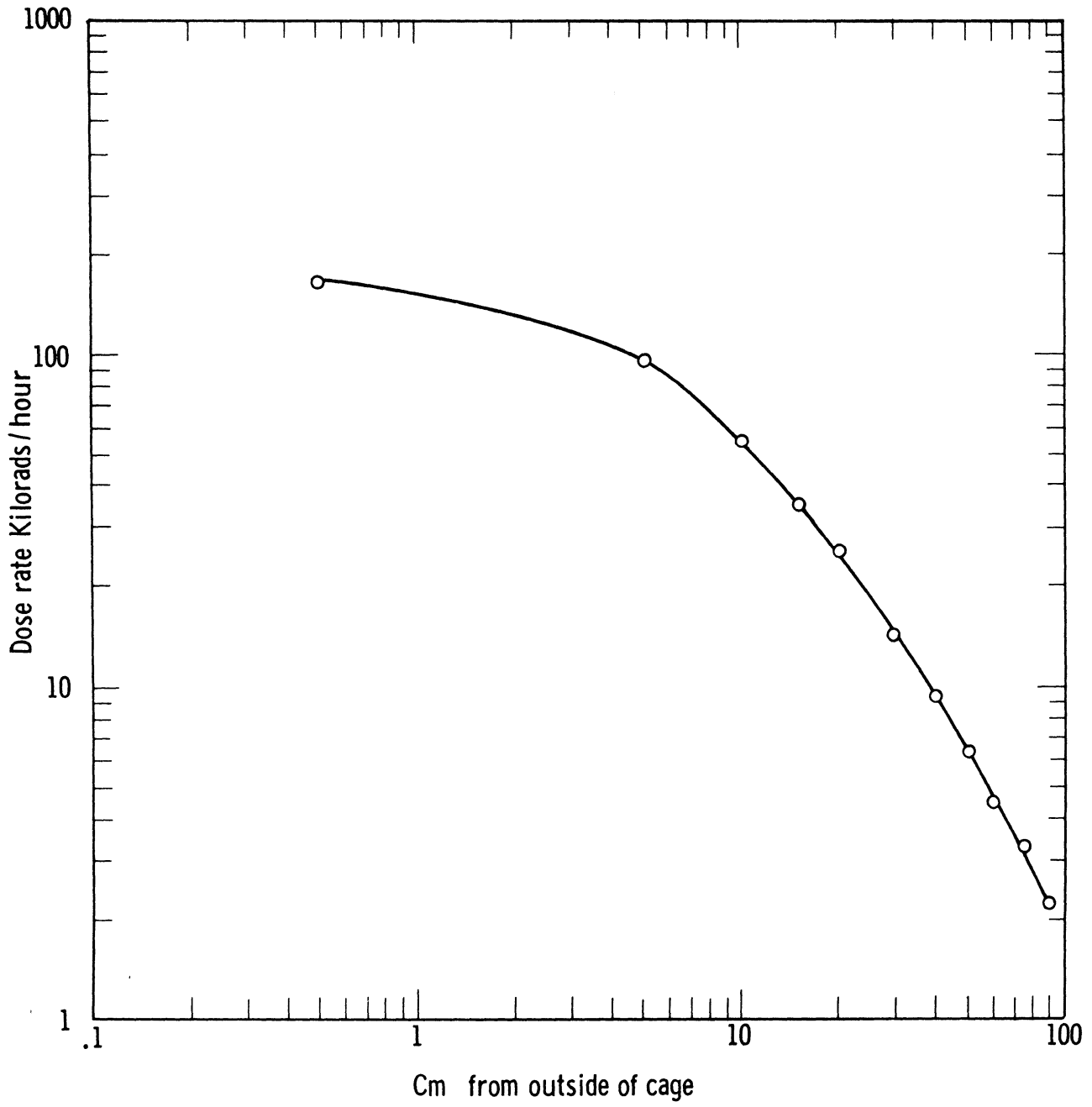Fig. A-10. Axial dose rates, Phoenix Memorial Laboratory cobalt-60 source (January 1, 1963).

Fig. A-11. Dose rates on midplane and outside of cage, Phoenix
Memorial Laboratory cobalt-60 source (January 1, 1963).

Decay Corrections

The dose rates provided by the cobalt-60 sources as reported should be corrected for the radioactive decay of cobalt-60 occurring anytime after the time the determinations were made. Table A-VIII gives the correction factors which should be applied after various time intervals of decay are listed. The same data are plotted in Fig. A-12.

TABLE A-VIII

DECAY CORRECTIONS

(Since the source calibrations were done on January 1, 1963, the corrections are to be applied to all measurements made after that time.)

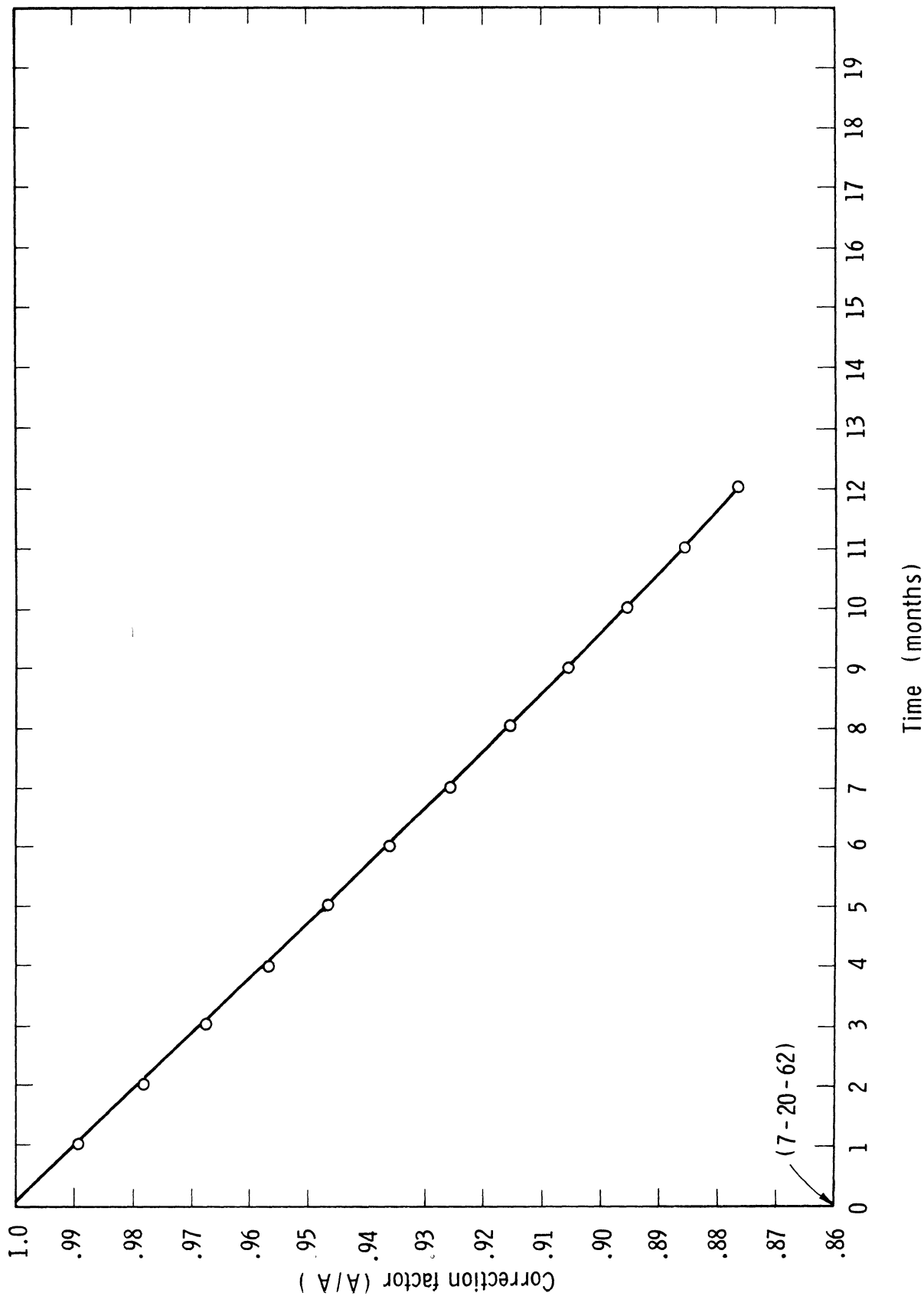| Time Interval (months of decay)	Correction Factor (A/A_0)
0	1.0
1	.9891
2	.9782
3	.9674
4	.9569
5	.9464
6	.9360
7	.9258
8	.9156
9	.9056
10	.8957
11	.8859
12	.8762
13	.8665
14	.8570
15	.8476
16	.8384
17	.8292

Fig. A-12. Correction factor vs. time (zero time, January 1, 1963).

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3. Kempe, L. L., Bonventre, P. F., Graikoski, J. T., and Williams, N. J., "Bacteriological Aspects of Ionizing Radiations in Food Processing," Atomic Energy Commission Report No. T1D-7412, 1956.

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