The Effect of Strain and Diet on the Thirty-Day Mortality of Acutely X-Irradiated Germfree Mice*

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Anemia, abnormal bleeding, disturbances in water and electrolyte metabolism and anorexia, as well as secondary infection, have often been considered as contributary to the pathogenesis of radiation sickness. Studies with germfree mice (1,2) have shown that death occurs after whole-body exposure to less than 1000 r of x-rays even in the absence of secondary infection. The significance of secondary infection in acute radiation death, as measured by the difference in $LD_{50/30}$ between germfree mice and their conventional counterparts, has varied from 45 r for Swiss-Webster mice (1) to 230 r for ICR mice (2).

The irradiation of different strains in different laboratories does not permit separate evaluation of the effects of strain and environmental variations on the response of germfree mice to x-irradiation. This report describes differences in the $LD_{50/30}$ of x-irradiated germfree and conventional mice of different strains and an increase in the $LD_{50/30}$ of germfree mice as a result of dietary supplementation.

MATERIALS AND METHODS:

All germfree and conventional mice were reared and maintained in the Biology Division of the Oak Ridge National Laboratory. Three strains of mice were studied. (1) Noninbred ICR mice whose ancestors were germfree ICR breeders supplied by the Lobund Laboratpry, University of Notre Dame. (2) Inbred RFM/Unf mice (3), and (3) Noninbred CF No. 1 mice whose ancestors were conventional CF No. 1 mice purchased from Carworth Farms, New City, N. Y., and were delivered by caesarian section into a sterile isolator and foster-nursed on a germfree ICR dam. The germfree mice were housed in plastic-film isolators (4) sterilized with ethylene-oxide and were maintained by a modification of the routine

procedures developed at the Lobund Laboratory. Surveillance for contamination consisted of periodic aerobic and anaerobic culture of feces and tissues for bacteria, fungi, and mycoplasma, examination of the intestinal contents and the skin for parasites, and serological examination for the following viruses: polyoma, reovirus 3, Theiler's virus, "K" virus, Sendai virus, pneumonia-virus-mouse, mouse adenovirus, and mouse hepatitis virus.

The conventional mice were either derived from germfree ancestors which had been removed from an isolator and placed in a breeder room with conventional mice more than one and one-half years prior to this experiment (ICR) or were removed from a colony which had always been conventionally reared (RFM and CF No. 1). Standard husbandry technics were used for the conventional mice except that the conventional ICR mice were fed the same sterilized diet as the germfree mice (autoclaved Purina Lab. Chow, 5010C). Microbiological studies were not carried out on these mice.

Ten to twelve-week-old germfree mice were irradiated in a sterile isolator while confined on a turntable under the x-ray beam (Fig. 1). The isolator housing mice to be irradiated, was kept in the same room as the source but the additional radiation amounted to less than 0.2% of the dose received on the turntable. Conventional mice were irradiated under identical conditions but were then removed from the isolator and returned to conventional surroundings. The GE Maxitron x-ray machine was operated at 300 kvp and 20 ma, with additional filtration of 1/4 mm Cu and 1 mm Al (HVL = 0.98 mm Cu.), at a dose rate of about 100 R/min. Most of the exposures were monitored with a continuously recording Radocon dosimeter. Following removal of the mice from the irradiation isolator, an unsterile Victoreen condensor dosimeter was introduced for determination of the dose rate. Cages were observed for dead animals twice daily. (F-1)

Supplementary diet preparations were purchased from Nutritional Biochemical Corporation, Cleveland, Ohio. The composition of the diet as published by the manufacturer is shown in Table I. All diets were filter sterilized by (T-I) filtration thru an asbestors filter pad.* The sterilized diets were passed into the isolator, diluted 1:1 with sterile distilled water and fed ad libitum as the sole source of fluids. Control mice were fed sterile distilled water. Mice were begun on the supplemental diet at 5 weeks of age and were maintained on the diet until the end of the experiment.

RESULTS:

The mortality curves for the three strains studied, ICR, CF No. 1, and RFM were determined by probit analysis and are shown in Figs. 2, 3, and 4. (F-2-4) Where data for males and females of the same strain were not significantly different, they were pooled for analysis. The germfree mice consistently had a higher $LD_{50/30}$ than their conventional counterparts (Table II), but the (T-II) difference in $LD_{50/30}$ between germfree and conventional mice varied with strain from 147 r for RFM males to 34 r for the CF No. 1. The $LD_{50/30}$ varied only one-half as much in the germfree (i.e., from 715 r to 789 r) as in the conventional mice (i.e., from 582 r to 729 r) but the order of decreasing strain radiosensitivity was different for conventional (i.e., RFM, CF No. 1, and ICR) and for germfree mice (i.e., CF No. 1, RFM, and ICR).

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*D-9 Sterilfo filter pads, F. R. Harmon & Co., Milldale, Conn.

The occurrence of two distinct and significantly different mortality curves for germfree ICR mice (Fig. 5) was discovered early in the course of these experiments. One curve is the compilation of data from experiments performed prior to 25 Dec. 1963. The other represents, and is typical for, all data accumulated after this date. The slopes of the mortality curves are different and the difference in the $LD_{50/30}$ values is significant, i.e., for germfree ICR mice irradiated before 25 Dec. 1963, 897 r, and after 25 Dec. 1963, 789 r. This difference, 106 r, is greater than the difference associated with strain in the germfree mice studied (i.e., 74 r). Conventional ICR mice irradiated during the same periods of time did not show a significant change in $LD_{50/30}$, i.e., 734 r before 25 Dec. 1963, and 729 r after 25 Dec. 1963. To determine whether a change in the quality of the diet could cause such a change in mortality, the regular diet, autoclaved Purina 5010 C, was supplemented with a filter-sterilized diet containing all of the essential nutrients known to be required for mice. Germfree ICR mice fed this supplement had a considerably lower mortality after x-irradiation than those not fed a dietary supplement (see Fig. 5). This "protective" effect of the dietary supplement does not appear to reside in any single group of essential nutrients since germfree ICR mice which received a supplement of one of the constituent groups of nutrients alone (i.e., amino acids fatty acids, vitamins, carbohydrates, or salts) had a mortality similar to that of unsupplemented mice (Fig. 6). (F-5) (F-6)

The influence of diet on weight loss after acute mid-lethal x-irradiation was studied by weighing supplemented and nonsupplemented germfree mice which had received various doses of x-radiation. At each radiation dose, the non-supplemented mice had lost more weight during the second, third, and fourth week following irradiation than their supplemented counterparts (Fig. 7). This difference (F-7)

in weight loss was also evident when supplemented and nonsupplemented germfree mice receiving an $LD_{50/30}$ dose of x-radiation (900 r for supplemented mice and 750 r for nonsupplemented mice were compared (Fig. 8). (F-8)

The mean survival time for germfree mice is significantly greater than that for their conventional counterparts in the three strains studied (Fig. 9). (F-9) Germfree CF No. 1 and ICR mice do not have different mean survival times over the LD_0-LD_{100} range, but the mean survival time for the germfree RFM mouse is from 3 to 6 days less than for germfree mice of the other two strains over the same dose range (Fig. 10). (F-10)

DISCUSSION:

The influence of strain on acute X-ray lethality in conventionally reared mice has been demonstrated in other laboratories (5,6). Some workers (7,8) have suggested that the genetic background determined the resistance of mice to acute X-radiation.

The results of our experiments demonstrate that the microflora plays a role in the determination of strain radiosensitivity, since the variation in $LD_{50/30}$ in the germfree mice is only one half that in the conventional mice and since the order of strain radiosensitivity is altered by the germfree state. Nonetheless, statistically significant variations in the $LD_{50/30}$ of different mouse strains were observed in germfree as well as in conventional mice indicating that although the microflora contributes to the radiosensitivity associated with strain, other determinantes are also present, possibly operating through the genetic background. This conclusion is further reinforced by data on the survival time of lethally X-irradiated germfree mice, where one strain, RFM, had a mean survival time from three to six days less than those of the other two strains.

The existence of two distinct mortality patterns for germfree ICR mice, as evidenced by the slope of the mortality curves, and differences in the $LD_{50/30}$ and in the mean survival time, suggests a change in some component of the isolator environment. Repeated attempts to identify contamination of the environment with microorganisms have always resulted in negative findings and although such negative results connot be conclusive, other possibilities must also be considered. Intentional alterations in the sterilizing time of the Purina diet used in this laboratory have been made, and although no such alterations coincided with the change in radiosensitivity of the germfree ICR mice, nutritional deficiencies caused by the loss of heat-labile nutrients after autoclaving have been considered a possible explanation. It has been shown that animals consuming a diet deficient in essential nutrients are less resistant to X-radiation than animals fed a complete diet (9,10). On the other hand, supplemental feeding of conventional animals during the period of radiation anorexia has not increased survival (11).

In our experiments, a filter-sterilized liquid diet containing all known essential nutrients required by the mouse, caused a decrease in the mortality and in the weight loss of acutely X-irradiated germfree ICR mice. Whether this "protective" effect of the supplemental diet is the result of a correction of a nutritional deficiency or simply the availability of an easily obtained, highly palatable food requiring little or no digestion, is not known at this time. Preliminary results, however, indicate that none of the major groups of essential nutrients alone is capable of modifying the mortality pattern of X-irradiated germfree mice, suggesting either that several nutrients are involved or that a complete diet is required.

The increase in radioresistance of supplemented germfree mice over their non-supplemented counterparts is consistent with the hypothesis that an adequate diet is unavailable to the germfree mouse. The possibility that the two radiation mortality patterns for germfree ICR mice are the result of a change in the diet is strengthened by the fact that dietary supplementation of germfree mice results in a mortality indistinguishable from that observed in the same mice irradiated before 25 December 1963, whereas unsupplemented mice show a mortality pattern characteristic for mice irradiated since that time (Fig. 7). The lack of change in radioresistance of conventional mice fed the autoclaved diet can be explained by the presence of bacteria in the feces of the mice, a ready source of dietary nutrients for coprophagic animals. Thus, the strain differences observed in the mortality of X-irradiated germfree mice may be due, at least in part, to variations in the combined effects of a submarginal diet and radiation.

SUMMARY:

Germfree and conventionally reared mice of the inbred RFM and non-inbred ICR and CF No. 1 strains were exposed at ∽ 100 r per minute to 500-1000 r whole-body X rays (hvl 1.0 mm Cu). In each strain, germfree mice had a higher $LD_{50/30}$ than their conventional counterparts, but the difference in $LD_{50/30}$ between germfree and conventional mice varied with strain from 147 r for RFM males to 34 r for CF No. 1 mice. The $LD_{50/30}$ of different strains varied only one-half as much in the germfree mice as in the conventional mice, and the order of strain radiosensitivity was different for conventional and germfree mice. Supplementation of the autoclaved diet with a filter-sterilized synthetic diet increased the $LD_{50/30}$ of germfree ICR mice from 790 to 900 r, changed the

slope of the mortality curve and the mean survival time, and decreased weight loss after irradiation.

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TABLE I - Typical Analysis of Nutrico, A Chemically Defined Liquid Diet. Numbers Indicate the Amount of Material per Liter of Diet.

AMINO ACIDS (IN GRAMS)

L Alanine	3.5	L Lysine HCl	6.5
L Arginine HCl	4.6	L Methionine	3.1
L Asparagine	1.75	L Phenyalalnine	3.1
L Aspartic Acid	5.0	L Proline	13.2
L Cysteine Ethyl Ester HCl	.09	L Serine	7.1
Glycine	2.2	Sodium L Glutamate	18.5
L Glutamine	7.3	L Threonine	4.3
L Histidine HCl H_2O	2.3	L Tryptophane	1.4
L Isoleucine	4.3	L Tyrosine Ethyl Ester HCl	8.6
L Leucine	6.8	L Valine	4.8

VITAMINS (IN MGS.)

Alpha Tocopherol Acetate	500.	2 Methyl-1-4 Napthoquinone	10.5
Ascorbic Acid	250.	Niacin	18.75
Biotin	0.15	p Amino Benzoic Acid	150.
Calciferol mcg.	17.5	Pyridoxine HCl	3.15
Calcium Pantothenate	25.0	Riboflavin	3.75
Choline Chloride	1250.	Thiamine HCl	5.0
Folic Acid	0.25	Vitamin A Acetate	25.
Inositol	125.	Vitamin B-12	0.05

TABLE I - (Contd.)

SALTS

Ammonium Molybdate $.4H_2O$ ..3 mg.	Monocalcium Fructose 1.6 Diphosphate ..... 25.25 gm.
Cobaltous Acetate $.4H_2O$ ..4.5 mg.	
Cupric Acetate $.4H_2O$ ..... 7.5 mg.	Potassium Hydroxide ..... 3.085 gm.
Ferrous Gluconate ..... 0.86 gm.	Potassium Iodide ..... 15 mg.
Glucuronolactone ..... 13.045 gm.	Sodium Bicarbonate ..... 5.45 gm.
Manganese Oxide ..... 3.085 gm.	Sodium Chloride ..... 2.0 gm.
Magnesium Acetate $.4H_2O$..130 mg.	Zinc Benzoate ..... 11 mg.

CARBOHYDRATES

Glucose ..... 342.85 gm.

FATTY ACIDS

Ethyl Lineoleate 100% ... 2 ml.

Tween 80 ..... 3-10 ml.

TABLE II - Numerical Results of $LD_{50/30}$ Determinations.* Large Numbers Indicate $LD_{50/30}$, Small Numbers Indicate The 95% Confidence Interval.

STRAIN	SEX	GERMFREE	CONVENTIONAL
ICR	♀	769.5 - 788.8 - 806.0	710.3 - 735.7 - 761.0
	♂	none irradiated	686.0 - 718.1 - 747.6
	♀ and ♂	——	709.9 - 728.9 - 747.1
CF No. 1	♀	705.5 - 719.6 - 733.3	634.5 - 695.7 - 756.9
	♂	697.6 - 710.4 - 722.9	583.7 - 665.1 - 746.5
	♀ and ♂	705.7 - 715.5 - 724.2	641.0 - 680.9 - 717.1
RFM/Un	♀	758.7 - 771.8 - 785.1	616.9 - 631.0 - 647.2
	♂	719.8 - 735.7 - 751.1	562.4 - 582.7 - 598.7
	♀ and ♂	Significant difference between sexes.	Significant difference between sexes.

*All mice irradiated after 25 Dec. 1963.

FIGURE LEGENDS

FIG. 1 - Placement of apparatus for the irradiation of germfree mice; irradiation isolator in the left foreground, housing isolator in the right background.

FIG. 2 - Thirty-day mortality of germfree and conventional ICR mice in relation to X ray dose. Data from males and females in four experiments are pooled; numbers indicate the size of each group; curves are fitted by probit analysis.

FIG. 3 - Thirty-day mortality of germfree and conventional CF No. 1 mice in relation to x-ray dose. Data from males and females in four experiments are pooled; numbers indicate the size of each group; curves are fitted by probit analysis.

FIG. 4 - Thirty-day mortality of germfree and conventional RFM/Un mice in relation to x-ray dose. Data from eight experiments are pooled; numbers indicate the size of each group; curves are fitted by probit analysis.

FIG. 5 - Effect of a filter-sterilized dietary supplement on radiation mortality in germfree ICR mice. Open figures and their corresponding curves are data accumulated before △—△ and after ⊙—⊙ 25 December 1963. Solid figures are data pooled from three experiments where supplemented ▲ and unsupplemented ● mice, housed in the same isolators, were exposed to various doses of x-rays. Numbers indicate the size of each group.

FIG. 6 - Effect of a filter-sterilized dietary supplement and its component parts on thirty-day mortality in germfree ICR mice exposed to 850 r of x-rays. Numbers indicate size of each group.

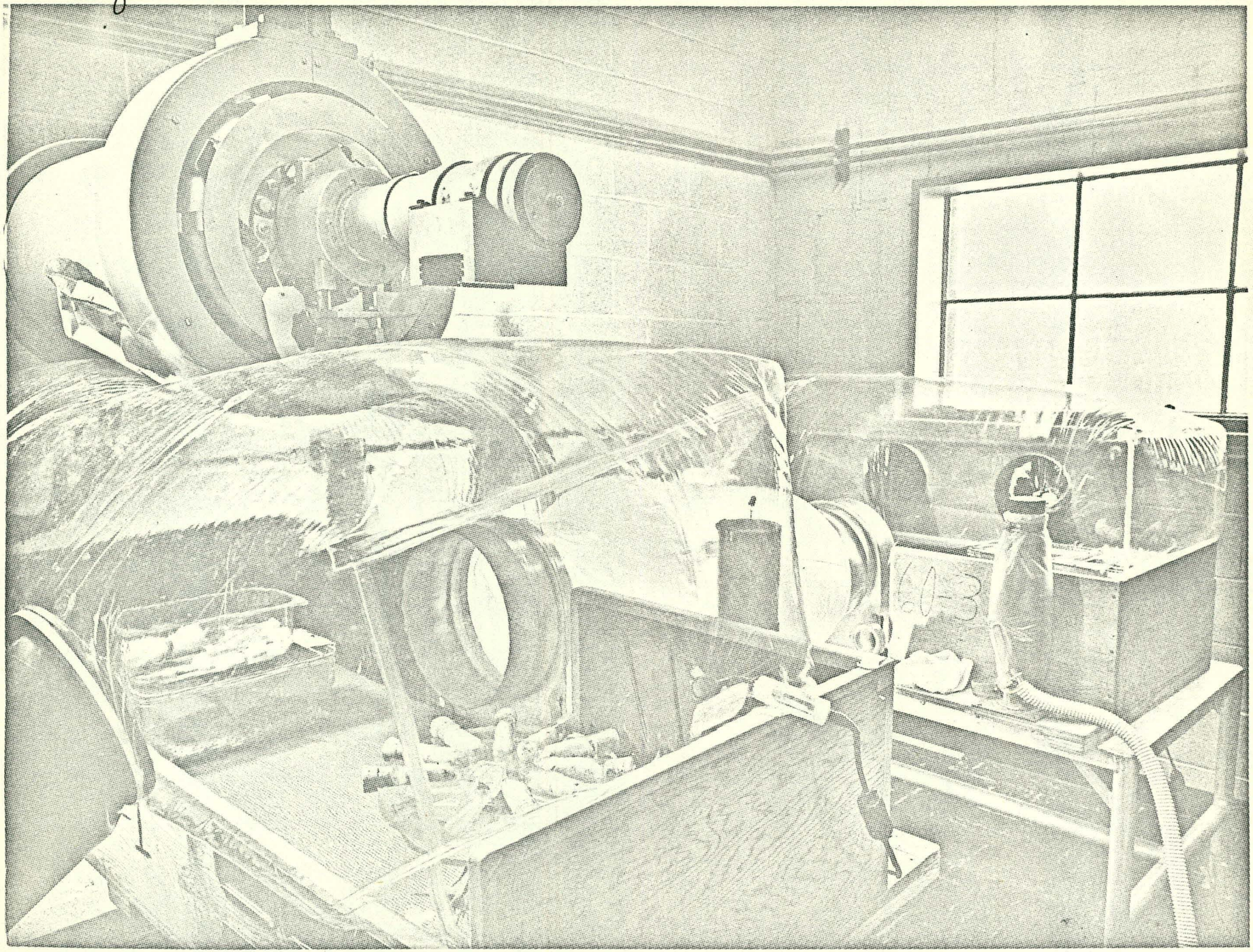
FIG. 7 - Effect of a filter-sterilized dietary supplement on weight loss in germfree ICR mice as a function of time after irradiation. Each point represents the mean of individual mouse weights taken in the isolator over the 30 day period after irradiation. Group initially consisted of 5-7 mice, but the size decreased with mortality.

FIG. 8 - Effect of a filter-sterilized dietary supplement on weight loss in germfree ICR mice, as a function of time, after an $LD_{50/30}$ dose of x-rays. Each point represents the mean of 3 to 4 individual mouse weights.

FIG. 9 - Mean survival time of x-irradiated germfree and conventional mice in relation to the observed mean thirty-day mortality. The standard error of the mean is shown for each point.

FIG. 10 - Mean survival time of x-irradiated germfree mice of various strains in relation to the observed mean thirty-day mortality. The standard error of the mean is shown for each point.

Fig. 1.



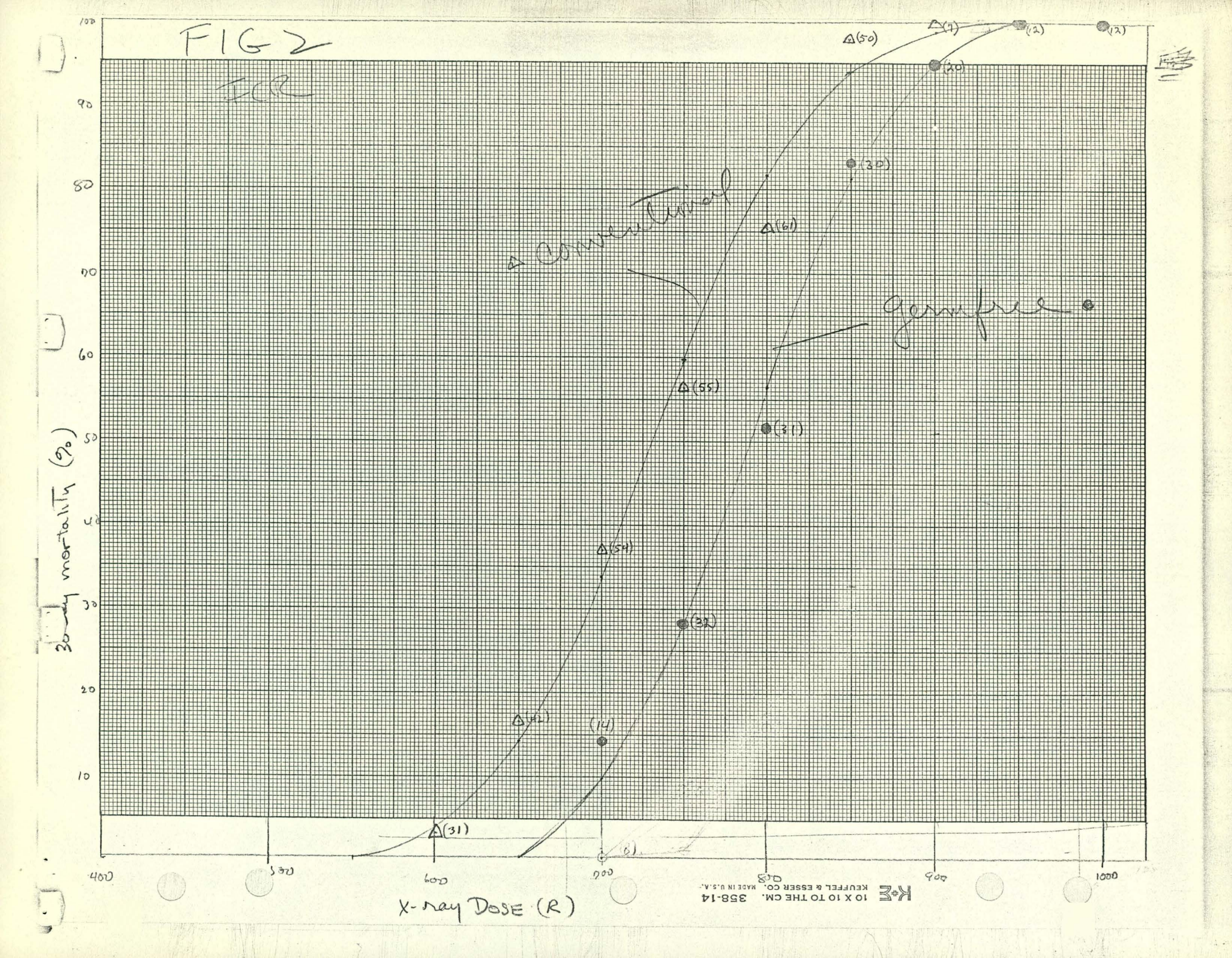


FIG 2
ICR
Δ Conventional
germfree
30 day mortality (%)
X-ray DOSE (R)
100
90
80
70
60
50
40
30
20
10
400
500
600
700
800
900
1000
Δ(50)
Δ(7)
(2)
(12)
(20)
(30)
Δ(61)
Δ(55)
(31)
Δ(54)
(32)
Δ(42)
(14)
Δ(31)
(6)
KE
10 X 10 TO THE CM. 358-14
KEUFFEL & ESSER CO. MADE IN U.S.A.

FIG 3

CF No. 1

Germfree

Conventional

X-Ray Dose

KEUFFEL & ESSER CO.

K&E 10 X 10 TO THE CENTIMETER 18 X 25 CM. 46 1510 MADE IN U.S.A.

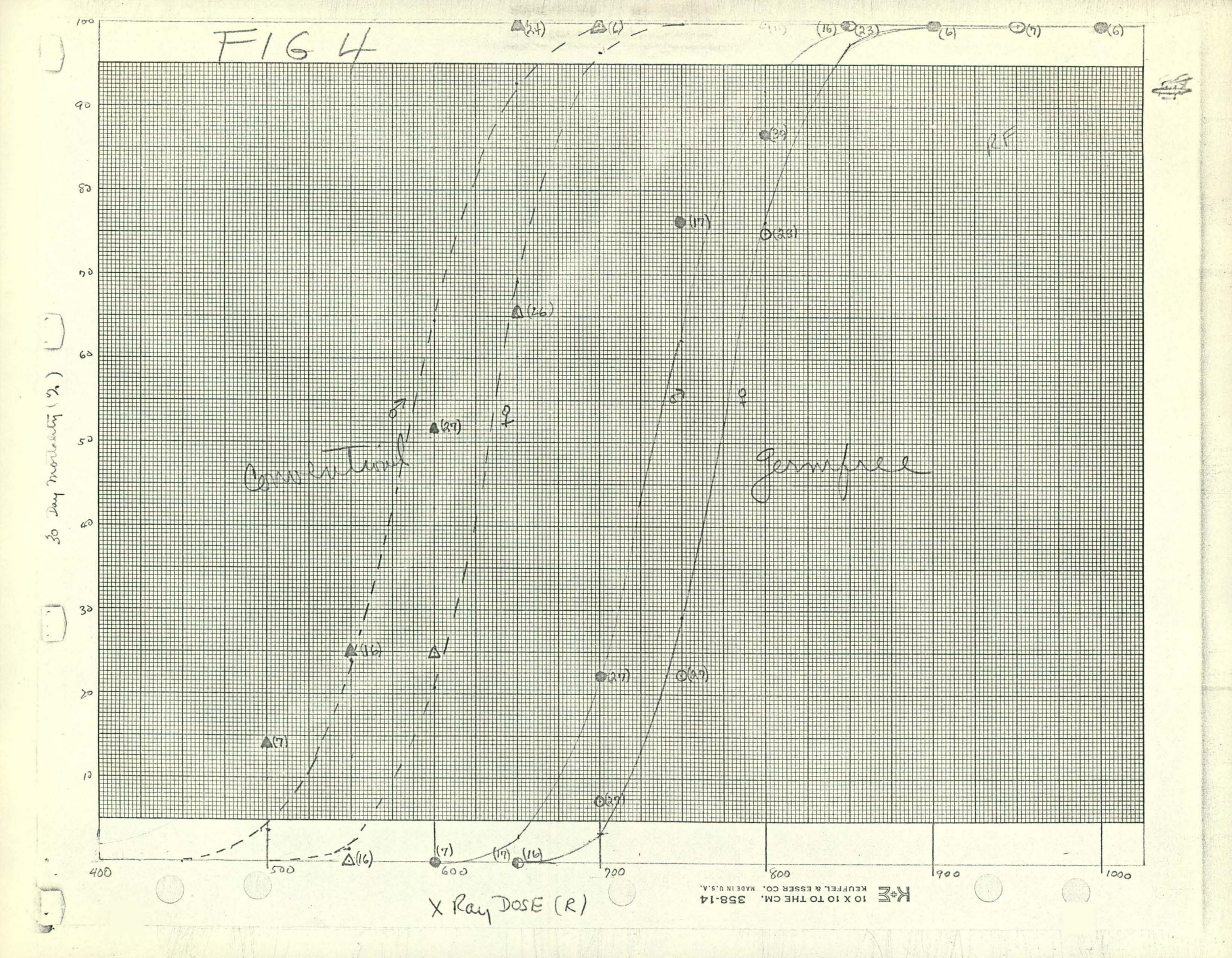


FIG 4
30 day mortality (%)
X Ray DOSE (R)
Conventional
Germfree
100
90
80
70
60
50
40
30
20
10
400
500
600
700
800
900
1000

Fig 5 Effect of a Filter-sterilized dietary supplement on radiation mortality in germfree FCR mice

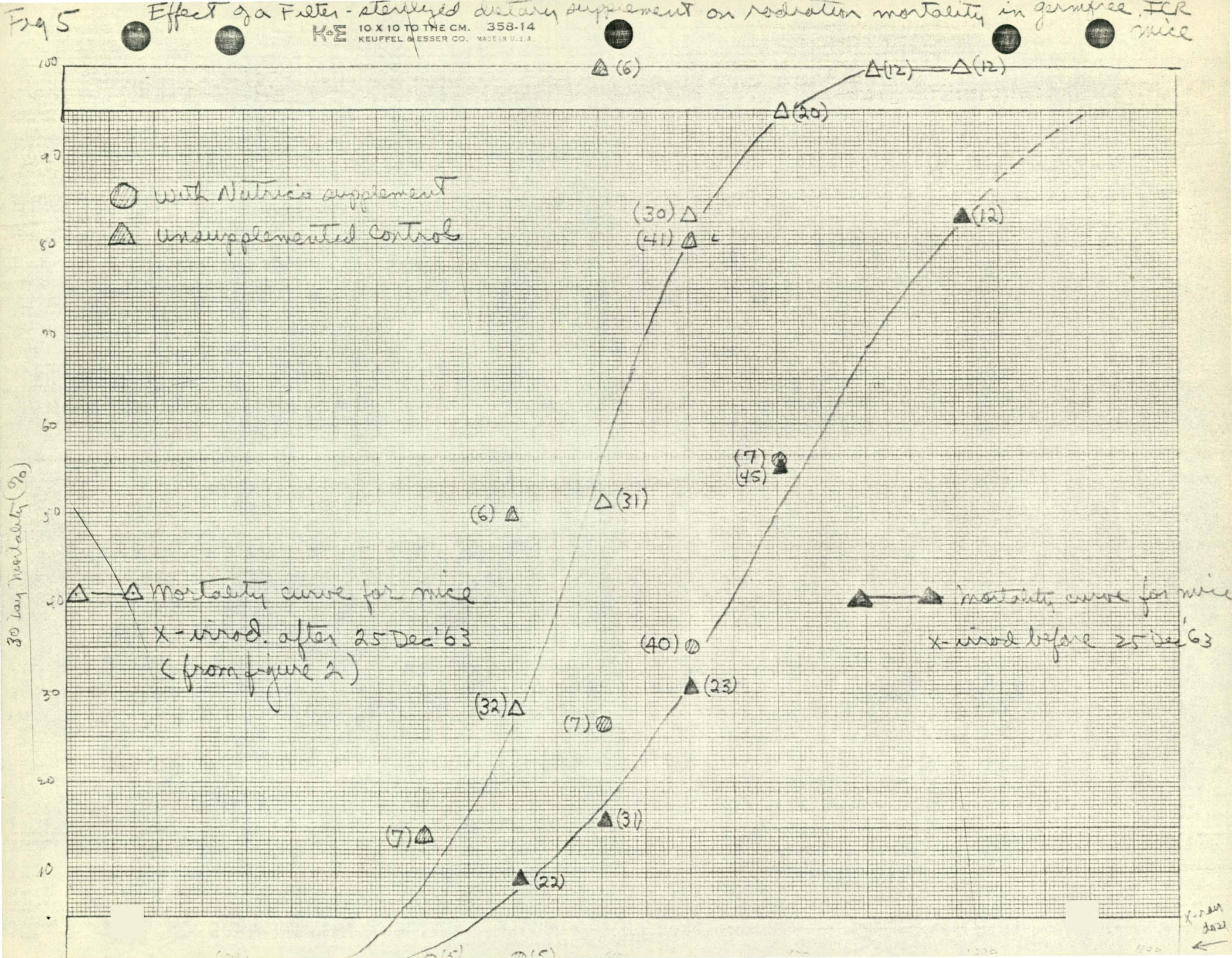


FIG 86

Effect of Nutrico and its component parts on Reduction mortality in germfree ICR mice.

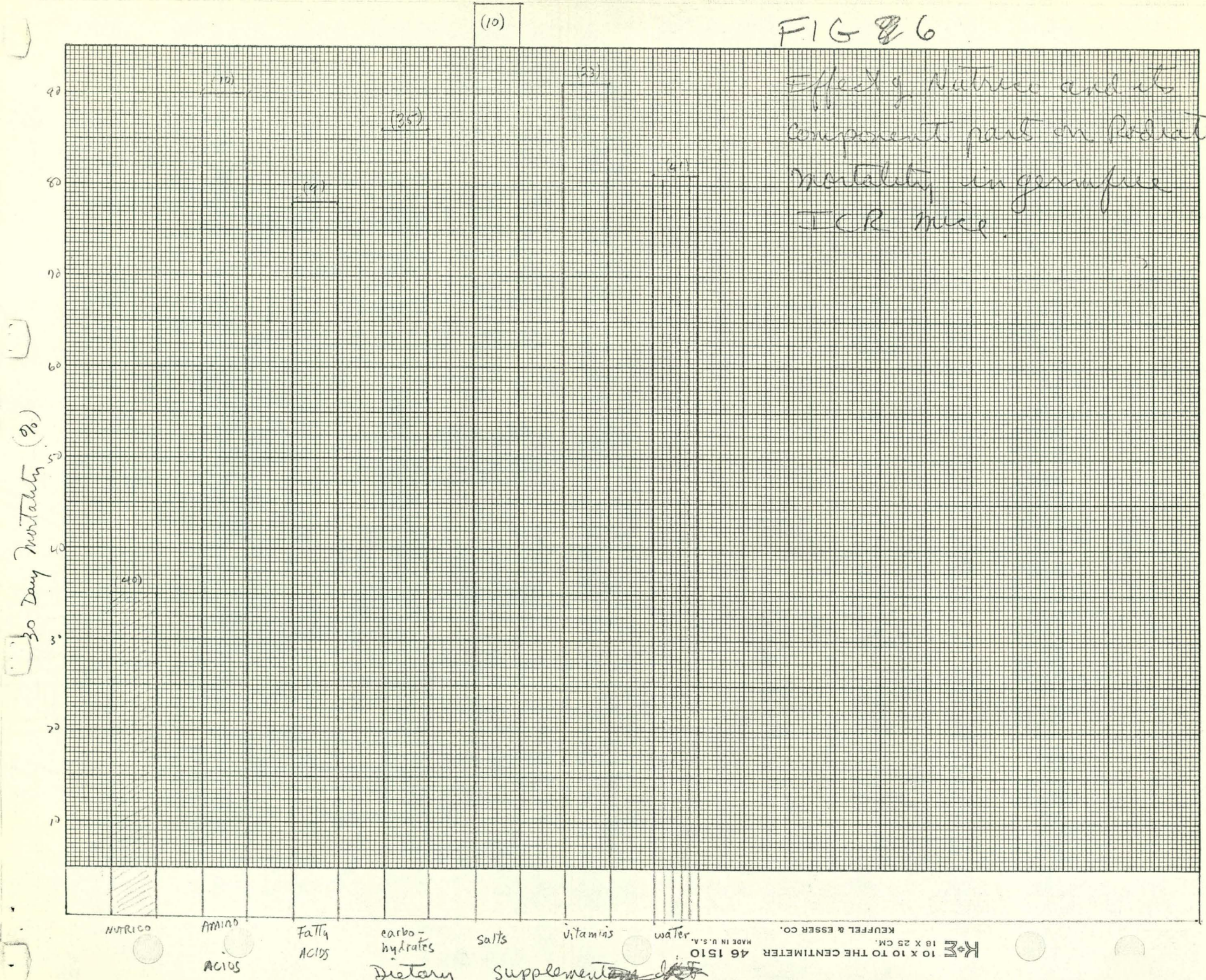


Fig. 47 (part 1)

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EFFECT OF A FILTER-STERILIZED DIETARY SUPPLEMENT ON WEIGHT LOSS IN GERMFREE ICR MICE AFTER X-IRRAD.

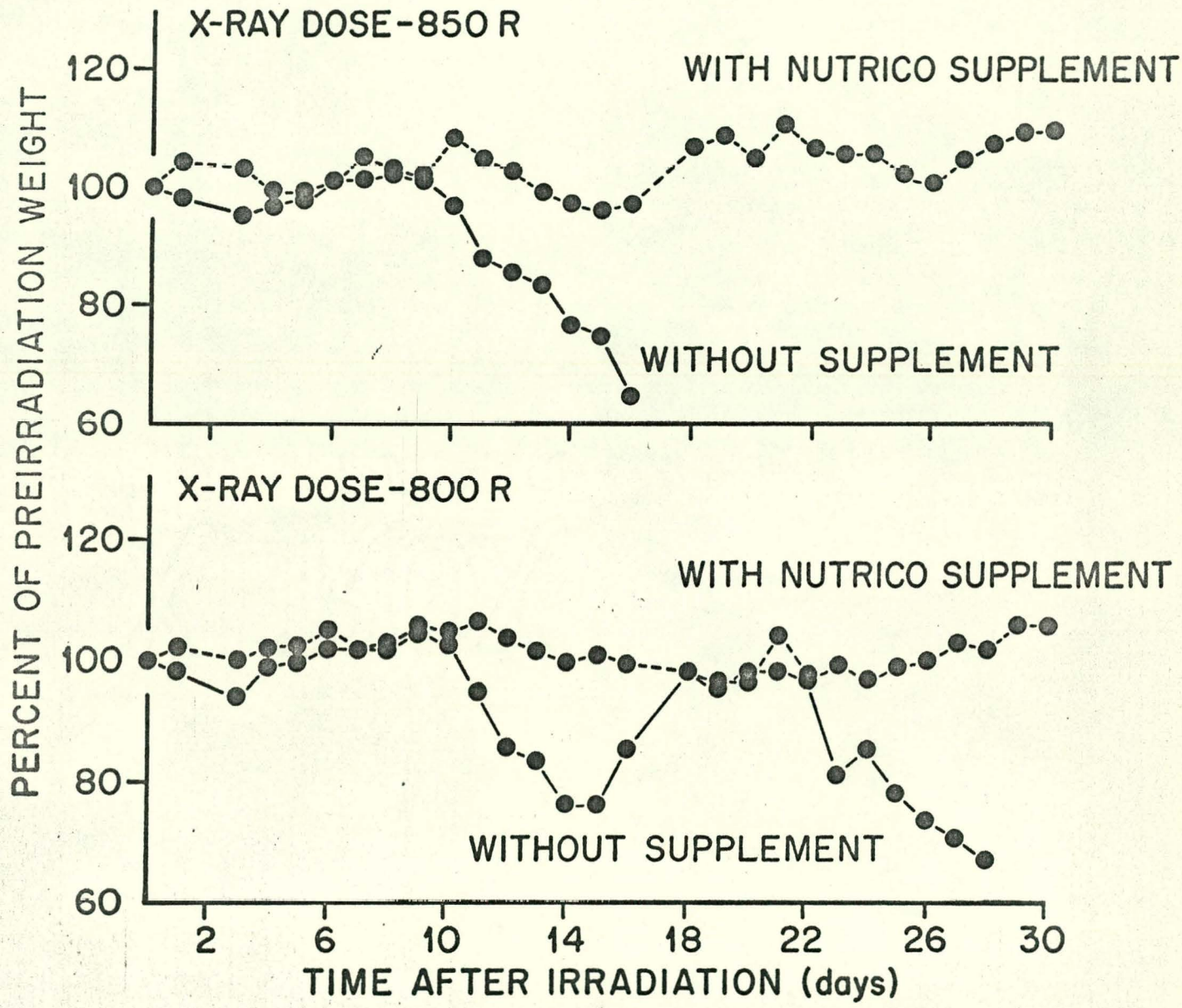

Fig #7 (part 2)

14,420

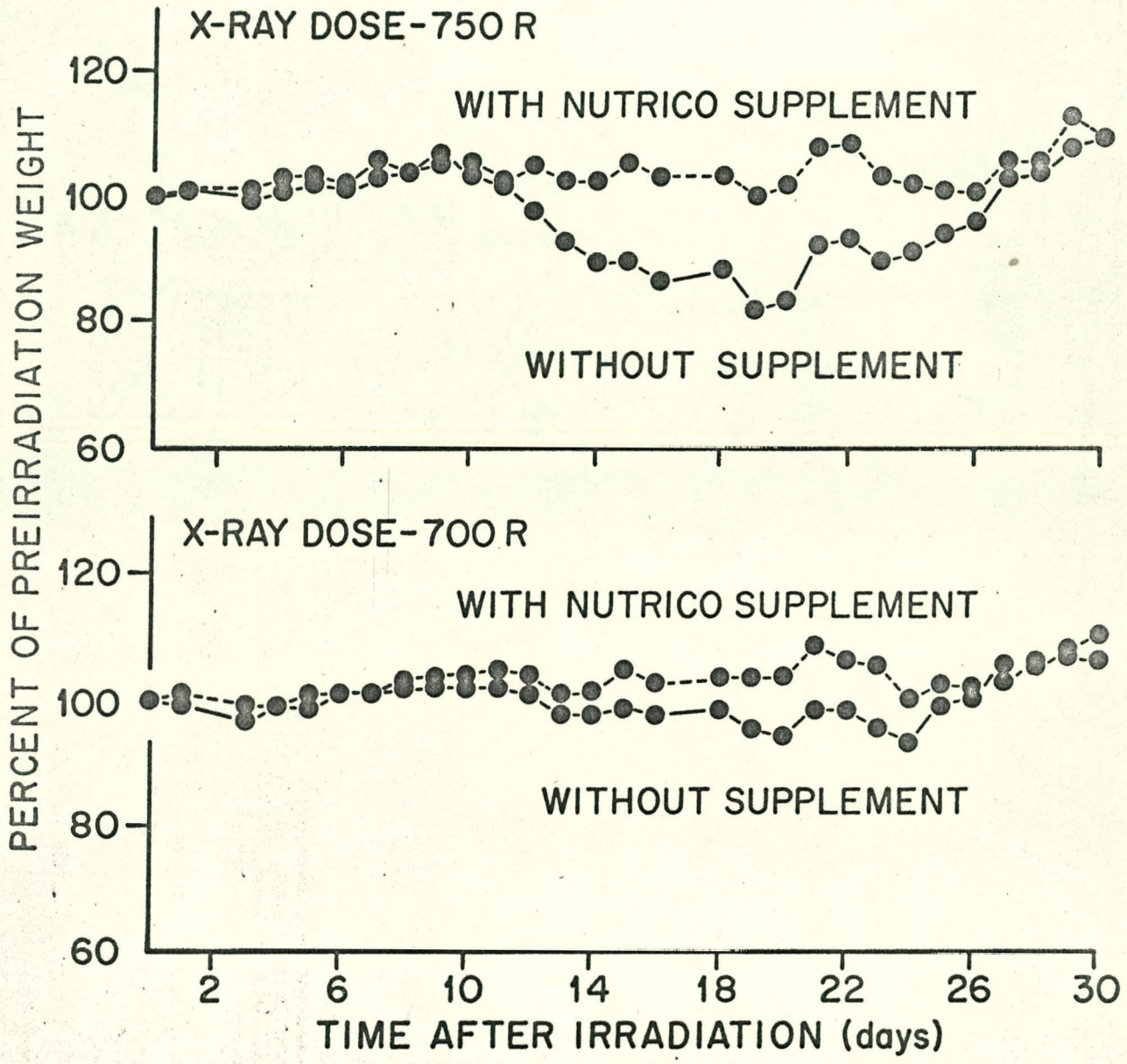

Fig 8

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EFFECT OF A FILTER-STERILIZED DIETARY SUPPLEMENT ON WEIGHT LOSS IN GERMFREE ICR MICE AFTER AN $LD_{50/30}$ DOSE OF X RAYS

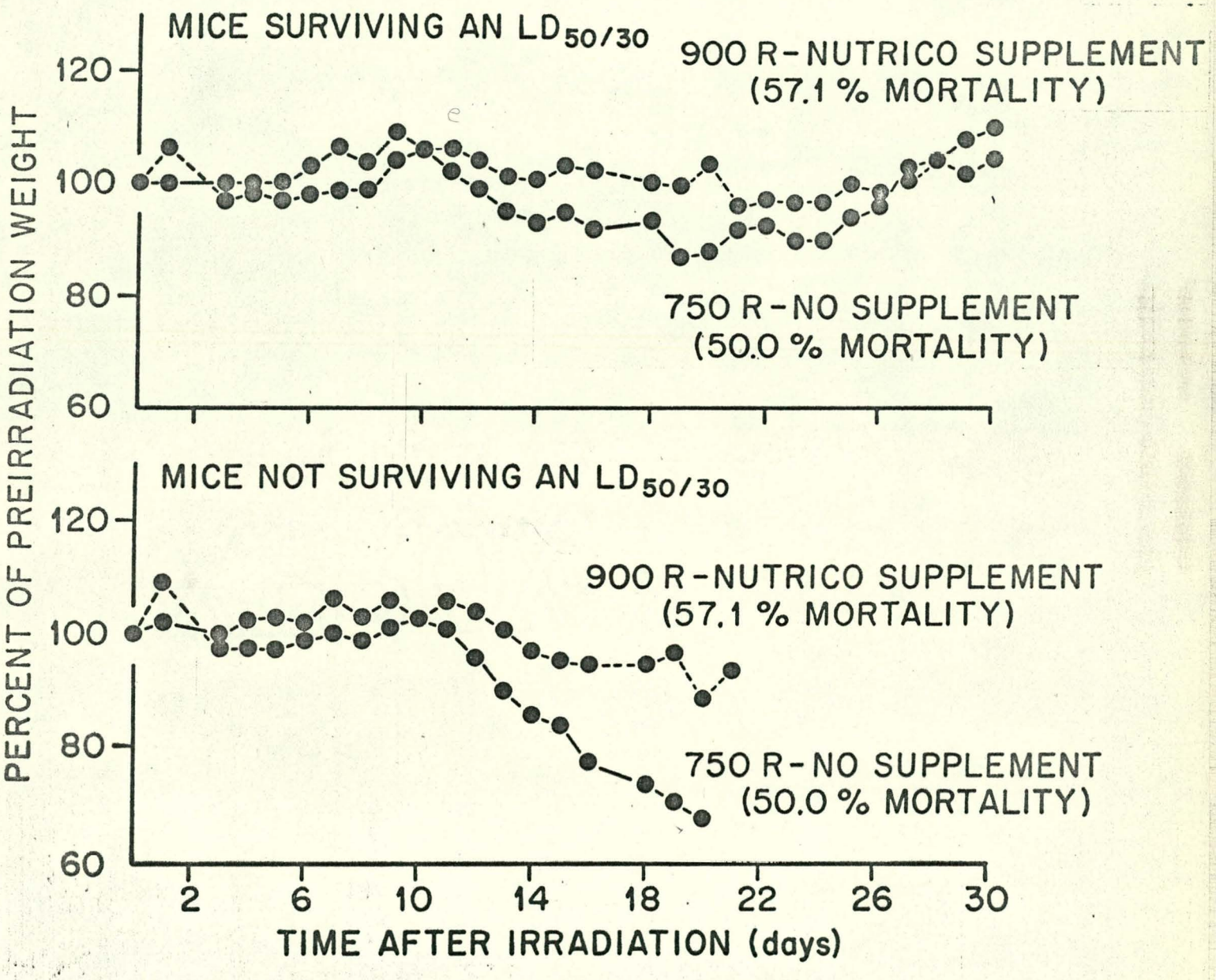

Fig 4 (part 1)

Fig 4 (part 1)

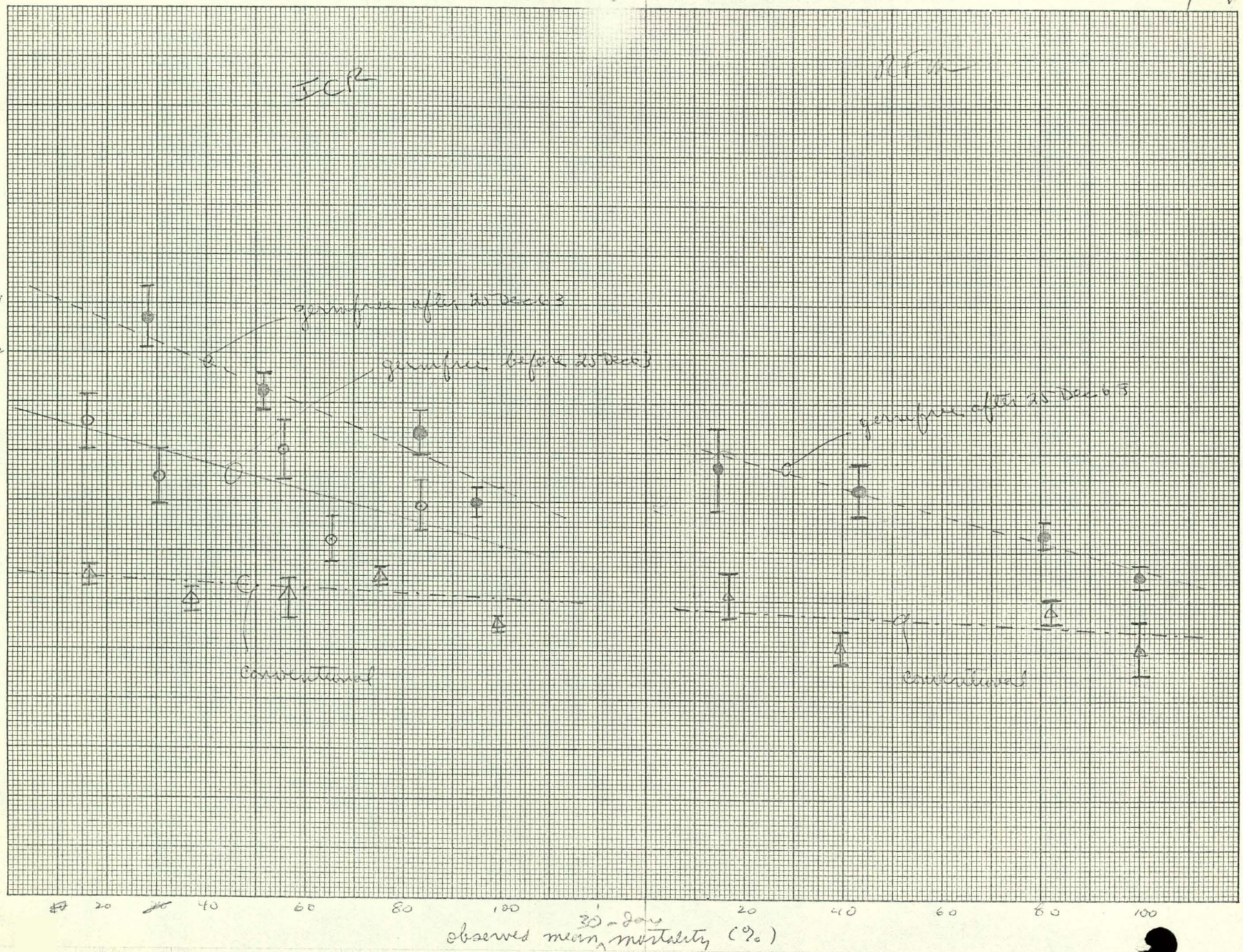

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Fig 4 (part 2)

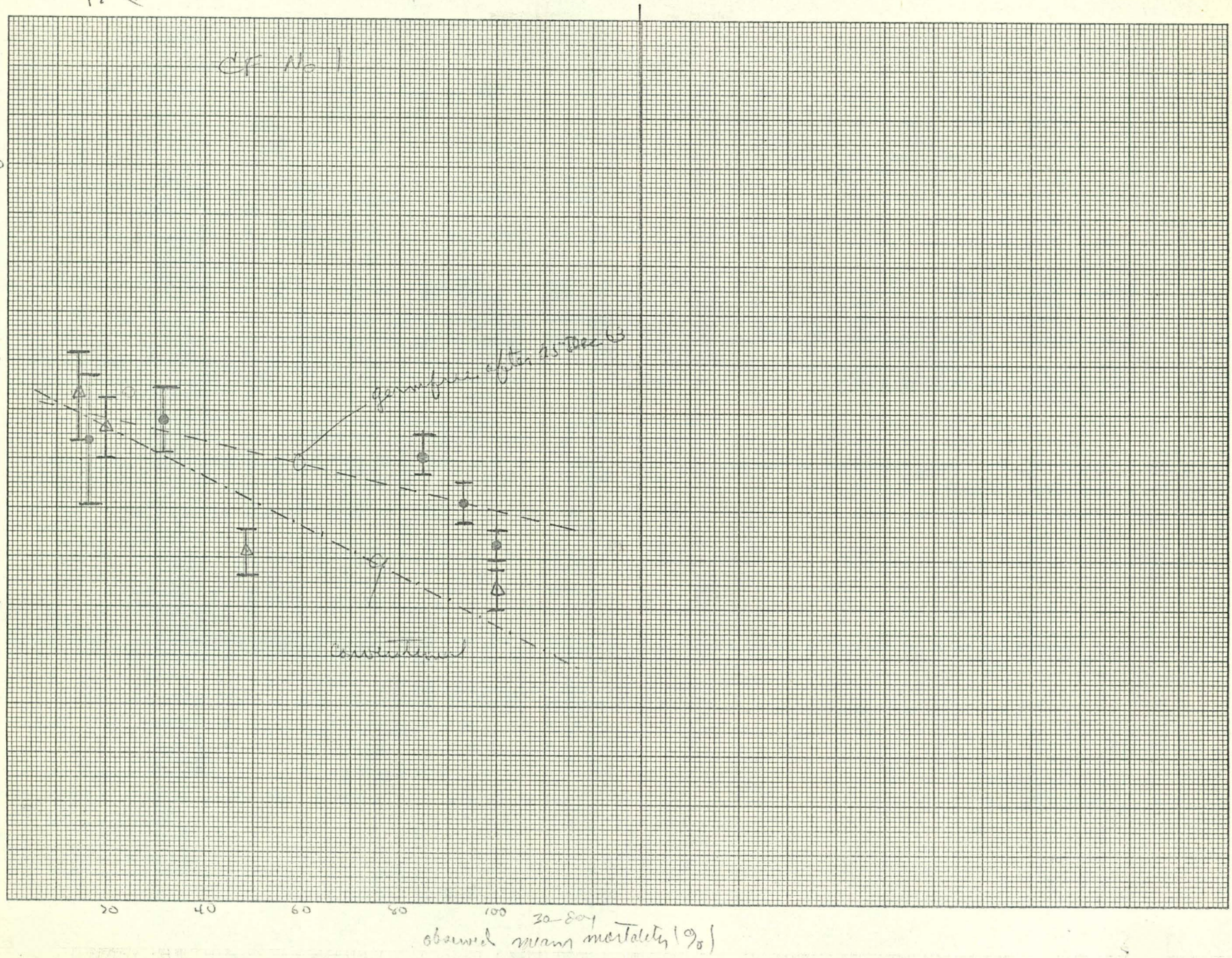

Fig 12.10