Project Number: 83-N5

1983 Annual Report

Project: Epidemiology and Control of Frost Injury to Almond Incited by Leaf Surface Ice Nucleation Active Bacteria

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Objectives During 1983:

1. To investigate the epidemiology of colonization of almond leaves, flowers and nutlets with ice nucleation active bacteria, including the assessment of sources of inoculum, of species and seasons populations of ice nucleation active bacteria predominating on frost sensitive plant parts.

2. Evaluate possible methods of application of antagonistic bacteria for use as biological control agents of frost injury.

3. Determine the most effective bactericides antagonistic bacteria, and ice nucleation active bacterial inhibitors to control frost injury and to determine the environmental parameters which influence their effectiveness.

4. To determine the supercooling of almond tissue as a function of time and treatments that alter the populations of ice nucleation activity of ice nucleation active bacteria.

5. To quantitate reductions of frost damage to almond in relation to reductions of populations of ice nucleation active bacteria on leaves and fruit of almond trees following application of bactericides and antagonistic bacteria to almond trees.

6. To select antagonistic bacteria from healthy almond leaves and flowers and to evaluate their efficacy for use as biological control agents of frost injury to almond.

7. To evaluate chemicals which inactivate ice nucleation active bacteria as frost control agents.

8. To evaluate the sources, activity and elimination of ice nuclei on dormant almond tissue.

9. To evaluate the copper sensitivity of bacteria isolated from almond tissues.

Interpretative Summary:

Extensive field trials were established in six different locations in the Spring of 1983. A large field trial, including twelve treatments, was established near Modesto, California. Field trials including sixteen different treatments were established at the West Side Field Station of the University of California, and more extensive numbers of trees were treated with bactericides or antagonistic bacteria on property owned by Teneco West near Snelling, CA. Plots on cooperating grower orchards were also established in Merced and Fresno Counties. Temperatures in the Spring of 1983 approached 32 F (0 C) in many locations, but in nearly all plots no significant frost injury resulted. Minimum temperatures of 29 F were encountered at Snelling, CA but with no resultant frost injury. Populations of ice nucleation active bacteria (nearly all were Pseudomonas syringae) in all field trial locations were very high during the Spring of 1983. Populations of ice nucleation active bacteria exceeded 100,000 per gram fresh weight of almond tissue in all locations, and approached 10^7 cells per gram fresh weight in some locations with a history of bacterial

blast during periods of maximum frost hazard. As in 1981 and 1982, a mixture of Maneb and cupric hydroxide conferred significantly better protection against bacterial populations and therefore frost sensitivity than did either material alone. Maneb alone gave no significant reduction of bacterial populations. Far better reductions of bacterial populations and resultant frost sensitivity to almond was achieved in orchards which had received dormant applications of copper compounds including Bordeaux or Kocide during dormant periods prior to bud break compared with trees sprayed at bud break only with bactericides. Since this has consistently been the observation, dormant applications of copper containing compounds appear to be an effective required treatment for reducing populations of ice nucleation bacteria on almond significantly in order to reduce frost hazard by subsequent sprays following blossoming. A large collection of ice nucleation active bacteria were made from all locations and are being tested for their sensitivity to copper ions. Preliminary data indicates that significant differences in resistance to copper ions exist among bacteria isolated from copper sprayed and non-copper sprayed almond trees. Highest levels of resistance to copper appears to exist among isolates of ice nucleation active bacteria from orchards with a long history of copper usage. Further work will be done to determine whether levels of resistance in these isolated bacteria are high enough to minimize the effectiveness of copper containing compounds in frost control or disease control. The enhanced bactericidal activity of copper containing compounds mixed with bis-dithiocarbamates such as Maneb may indicate that this is the case. A new technique has been developed to measure the supercooling point of almond tissue. Supercooling point, the lowest temperature below 32 F any plant part can be cooled before ice nucleation and therefore frost damage to the plant can occur, can be directly related to the frost sensitivity of these plants under field conditions. The supercooling point of untreated almond tissues increase from about -4 C at bud break to only about -2 C (29 F) after full bloom. This increase in the supercooling point also parallels the increase in populations of ice nucleation active bacteria showing that they are responsible for the frost sensitivity of almond tissue. The lower supercooling point of almond tissue from orchards with a history of bacterial blast also show that fairly large differences in frost sensitivity occur among almond orchards. Approximately 2-4 F frost

protection has been observed on plants treated with bactericides or antagonistic bacteria as measured by the estimations of supercooling points of tissue. Small numbers of nonviable bacterial ice nuclei may exist on dormant almond tissues and studies are underway to determine whether these can be eradicated with dormant applications of nucleation inhibitors.

Experimental Procedures:

Many of the experimental procedures used in this study during 1983 were similar to those reported in 1980, 81 and 82 annual reports, or in the technical publication to be published in January 1984 in the Journal of American Society of Hortscience. Bactericides were applied at label rates in the case of cupric hydroxide or at 150 parts per million and 75 parts per million active ingredient of a mixture of streptomycin and Terramycin respectively. All bactericides sprays were applied with added surfactant Triton CS-7 with either a handgun sprayer at approximately 300 gallons per acre or with a speedsprayer at approximately 200 gallons per acre. Antagonistic bacteria were applied to almond trees at about 2% bloom at a concentration of about 10^8 cells per milliliter with a backpack mist blower. Approximately 1 gallon per tree bacterial suspension was applied. Other chemicals including phosphoric acid and Hyamine 2389 were applied with a handgun sprayer to runoff. Similarly bacterial nucleation inhibitors applied to dormant trees were applied to runoff (approximately 50 gallons per acre) with a handgun sprayer. Bacterial populations on almond leaves, flowers and nutlets were quantitated by removing bacteria from the surface of the leaves by emersing them in sterile phosphate buffer followed by sonication for 8 minutes. Dilution plating of bacterial suspensions was then done on King's Medium B or King's Medium B containing appropriate antibiotics for identification of an antibiotic marked antagonistic bacteria. The numbers of ice nuclei on almond tissue was measured from these same leaf washings by placing a collection of forty or more droplets of leaf washing solution on the surface of aluminum sheets held at -5 C or -9 C. The number of ice nuclei active per milliliter and thus per gram of leaf tissue could be determined from the fraction of droplets which remained unfrozen at these temperatures. Supercooling point of almond, leaf, flower and nutlet tissues were also determined during

1983. The distribution of freezing temperatures of almond tissue was determined by placing 40 or more plant parts in tubes of ice nucleus-free water at -2 C or warmer. The temperature of the tubes was then slowly cooled at 0.5 C temperature intervals, and at each interval the number of tubes which froze was determined. From the normal distribution of freezing temperatures observed, the median supercooling point of a given population of leaves, flowers or nutlets was determined by computer-directed algorithms. The copper sensitivity of randomly isolated ice nucleation active bacteria from almond tissue from various sources was determined by growth on nutrient agar containing various concentrations of copper sulfate in distilled water containing various concentrations of cupric sulfate and by a radial diffusion assay in yeast extract caseamino acid agar pH 7.2.

Results:

Almond leaves, flowers and nutlets were colonized with very high numbers of ice nucleation active bacteria during 1983. Over 100,000 ice nucleation active bacteria per gram of leaf and flower tissue was observed on trees during periods of maximum frost hazard on untreated trees in several locations (Fig. 1, Table 1, Table 2 and Table 3). Populations of ice nucleation active bacteria on untreated trees reached their maximum populations very shortly after first bloom. Populations increased for one to two weeks and then maintained a constant level for approximately three more weeks and then slowly declined toward the end of April. Total populations of bacteria remained fairly constant and were generally higher than about 10^6 cells per gram fresh weight at all times. The number of ice nuclei on untreated almond trees paralled the increase in the numbers of ice nucleation active bacteria on these trees (Figure 1, Table 1, Table 2, Table 3). Approximately 1 cell in 180 were active in ice nucleation at temperatures of -5 C or warmer while on almond trees (Fig. 1, Table 1, Table 2, Table 3). The populations of ice nucleation active bacteria were reduced approximately 40-fold by treatment with cupric hydroxide (KOCIDE 101) alone (Fig. 2). The number of ice nuclei active at either -9 or especially at -5 C were also reduced approximately 50-fold by treatment with cupric hydroxide alone (Fig. 1, Table 1, Table 2, Table 3). However, when Maneb at label rate was added as a tank mix to KOCIDE 101, the

reduction of ice nucleation active bacteria and bacterial ice nuclei was dramatic. It ranged from approximately 300-fold to 10,000-fold compared to untreated control plants (Fig. 3). The total number of bacteria on plants treated with a mixture of KOCIDE 101 and Maneb was also lower than on untreated trees. Thus clearly in 1983 a synergistic effect of application of KOCIDE 101 and MANEB was observed. It also appears that the synergistic effect seen with such mixtures is not specific only to that of Pseudomonas syringae but is in general an enhanced bactericidal effect of cuprichydroxide. Whereas a mixture of streptomycin and Terramycin was more effective than cupric hydroxide alone in reducing in bacterial populations and specifically populations of ice nucleation active bacteria, they were not as effective as a mixture of cupric hydroxide and Maneb (Fig. 4). Populations of ice nucleation active bacteria on plants treated with streptomycin and Terramycin were reduced approximately 100-fold at all dates compared with untreated plants.

Applications of copper fungicides during a dormant phase in addition to applications starting at bud break greatly increased the bacterial control seen with such subsequent applications. It can be seen from examination of Tables 1, 2 and 3 that application of KOCIDE only in the dormant phase yielded insignificant reduction of ice nucleation active bacteria on most dates. However when cupric hydroxide was applied weekly or at ten-day intervals starting at bud break to trees with and without dormant applications of KOCIDE 101, control of ice nucleation active bacteria and bacterial ice nuclei was significantly better on trees which received both a dormant and subsequent weekly applications starting at bud break compared with trees sprayed only at bud break and later.

Several non-ice nucleation active bacteria applied to almond trees at first bloom significantly colonized almond tissue and reduced subsequent colonization of this tissue by ice nucleation active bacteria (Figures 5, 6, 7). The most effective bacterium singly or in combination with other bacteria were the isolates C13-12 and C30-11 which were mutants of Pseudmonas sryingae and Erwinia herbicola respectively which no longer catalyze ice formation. These bacteria readily colonized almond tissue and reduced populations of ice nucleation active bacteria and bacterial ice

nuclei approximately 45-fold compared to untreated trees. These bacteria colonized treated almond trees for at least 30 days following application and reduced frost sensitivity of these trees accordingly. Trees treated with a dilute solution of phosphoric acid also exhibited lowered populations of both ice nucleation active bacteria, but primarily bacterial ice nuclei active at -5 C (data not shown). Mild phytotoxicity was observed in 1983 with applications of dilute phosphoric acid.

The supercooling points of almond spurs closely followed the reductions in populations of ice nucleation active bacteria and of bacterial ice nuclei on trees. Untreated control trees generally had the highest supercooling point (approximately -1.9 to -2.3 (Figure 9, Tables 4, 5)). The supercooling points of almond tissue was observed to be normally distributed as evidenced by Figure 8. When the cumulative percent of detached almond spurs frozen as a function of decreasing temperature was regressed against temperature, linear relations indicative of normal distributions were invariably observed. Because of the normal distribution of freezing points, estimates of median supercooling points could be readily obtained from regression analyses of the distribution of freezing points. Trees treated with antagonistic bacteria C1213-12, C30-11 (Squares) or with a mixture of KOCIDE plus Maneb froze approximately 0.8 to 2.3 C colder than that of untreated almond tissues (Fig. 9). Similarly the supercooling point of almond trees treated with cupric hydroxide on a dormant as well as a weekly basis following bud break were lower than that of untreated trees or trees treated only starting at bud break with cupric hydroxide.

Discussion:

Worked done during 1983 indicates the feasibility of significantly reducing the supercooling point of almond tissue and thus the ability of almond to escape damaging ice formation under field conditions, by reducing the population of ice nucleation active bacteria by both biological and chemical means. Several non-ice nucleation active bacteria were particularly adept at colonizing almond tissue following a single spray early in the spring. Efficient colonization of trees by these bacteria

reduced epiphytic colonization of this tissue with ice nucleation active bacteria and therefore reduced the supercooling point compared to untreated trees. Certain almond orchards apparently have higher populations of ice nucleation active bacteria than others and therefore might be considered to be at much more frost hazard. Reductions of supercooling points in these orchards were lower than in orchards which had lower overall bacterial populations. Therefore more work is needed to determine the variability of epiphytic populations of ice nucleation active bacteria on different almond orchards in different geographic areas or having different management practices. Whereas copper containing fungicides alone are fairly effective at reducing populations of ice nucleation active bacteria, a mixture of KOCIDE 101 and Maneb was significantly better at reducing these populations than either alone. These results and other data not yet tabulated suggest that some level of copper tolerance among ice nucleation active bacteria may exist among field populations of these bacteria. These bacteria probably do not represent true resistance to copper but have adapted to tolerate low levels of copper ions present on plant tissue after application of cupric hydroxide. This low level tolerance may reflect a somewhat higher ability to restrict migration of copper ions into the cells. Other workers have also shown that the addition of chelating agents such as bis-dithiocarbamate fungicides can increase the ability of copper to kill copper tolerant strains of these bacteria. This phenonmenon of copper tolerance needs further investigation on almond tissues and will be the subject of more intense investigation during 1984. Use of chelating agents such as Maneb to improve the control of bacterial populations or frost control and the control of bacterial blast or canker of almond is also warranted. Since Maneb also represents a fairly effective fungicide which can be substituted for several of the fungicides now used on almond, this would seem to represent no increased cost to growers and might greatly improve their disease and frost control practices. Evidence is accumulating that low numbers of ice nuclei of fairly high activity can overwinter on dormant almond tissue. Preliminary evidence does indicate however that these bacteria are sensitive to inactivation with currently investigated ice nucleation inhibitors such as phosphoric acid as well as high levels of soluble copper sulfate, sodium hypochlorite or guanidine

hydrochloride. This work will be further evaluated during 1984 as a further management practice that might reduce the use of copper based fungicides during dormant applications.

TABLE 1

Bacterial and ice nuclei populations on NePlus ultra almond on April 19 sprayed at different times with bactericides

Treatment	log cells/g. fr. wt.				log ice nuclei/g.	
	Total	Yellow	Fluorescent	ice nucleation active	-5 C	-9 C
Kocide dormant and pink bud	4.64	4.42	3.71	0.97	0.00	0.36
Kocide pink bud only	6.42	5.85	4.10	4.52	1.12	1.49
Check	6.57	4.26	5.63	4.22	1.57	1.91
F (treatment)	42.56	0.82	6.12	8.76	11.03	18.42
LSD 5%	0.52	NS	1.31	2.12	0.78	0.59

Table 2

Bacterial Populations and Populations of Ice Nuclei
on Ne Plus Ultra Almond on March 3, 1983
Sprayed at Different Times with Bactericides

Treatment	log cells/g fr. wt.				log ice nuclei/g	
	Total	Yellow	Fluorescent	Ice nucleation active	-5 C	-9 C
Kocide dormant + pink bud	5.57	1.55	4.68	4.64	1.12	2.44
Kocide pink bud	5.63	4.53	4.36	4.83	2.05	3.29
Check	5.66	4.30	3.77	5.11	1.43	2.97

Table 3

Bacterial populations and numbers of ice nuclei on almond spurs treated at different times and frequencies with copper fungicides

Fresno March 7, 1983

Treatment	Bacterial Populations log(cells/g fr. wt.) Total	Fluorescent	log ice nuclei/g fr. wt. INA	-5C	9C
Kocide bud break only	5.24	4.56	4.53	4.31	4.75
Kocide weekly	4.29	3.72	3.79	1.79	2.97
Kocide dormant only	5.46	4.47	4.41	3.06	4.24
Kocide dormant+bud break	4.93	4.14	3.88	3.64	4.23
Kocide dormant +weekly	4.61	3.83	2.99	1.23	2.43
Kocide twice dormant only	4.81	4.17	4.52	2.92	3.63

Table 4

Supercooling point of almond spurs treated with bactericides

Clovis march 3, 1983

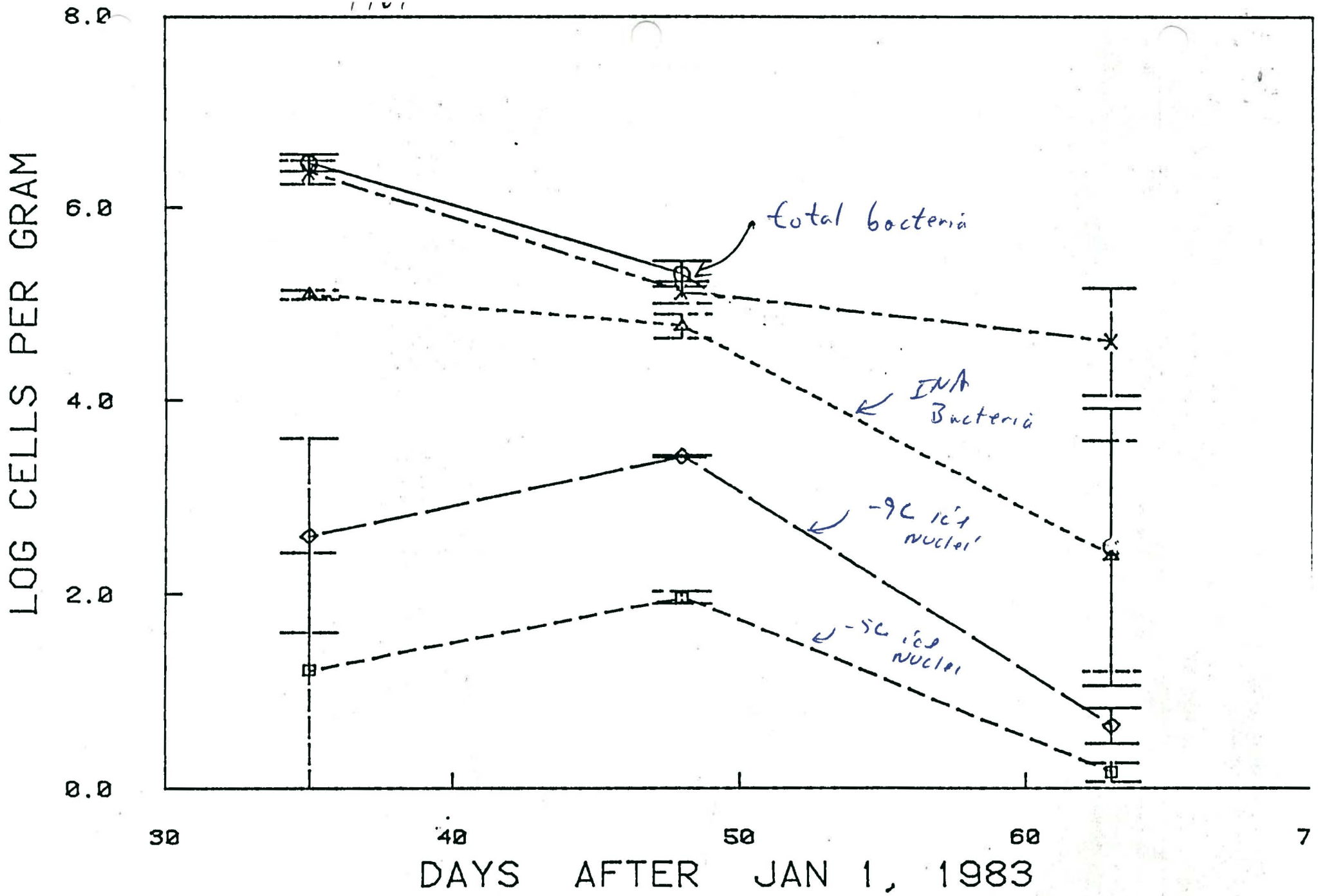
Treatment	Supercooling Point (C)
NePlus -Kocide dormant +weekly	-2.73
NePlus -Kocide weekly	-2.43
NePlus - Check	-2.02
Mission -Kocide dormant+weekly	-2.53
Mission -Kocide weekly	-2.20
Mission - Check	-2.10

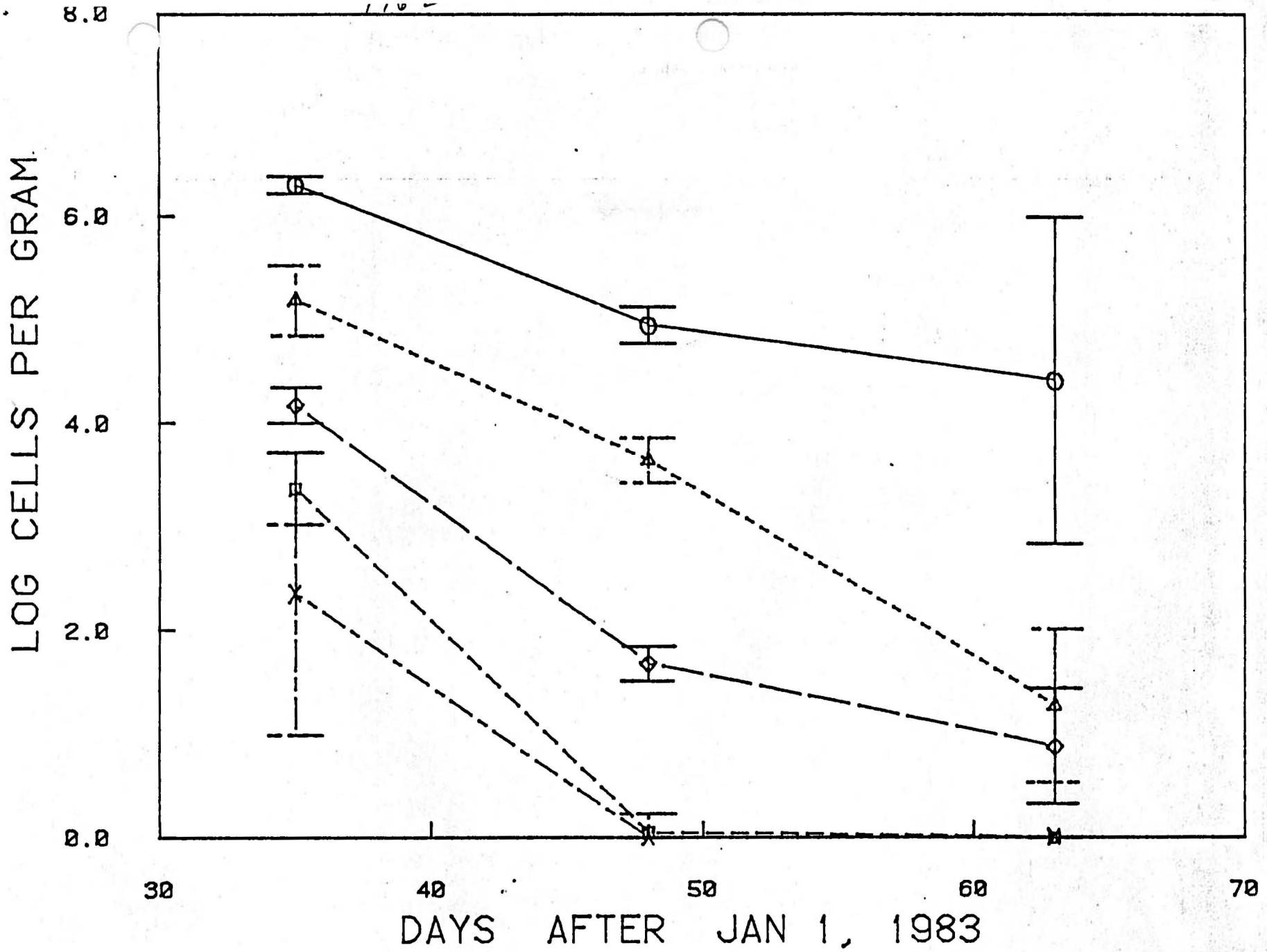
Table 5

Supercooling point of almond spurs treated with bactericides

Clovis April 19, 1983

Treatment	Supercooling Point (C)
Mission -Kocide dormant+weekly	-2.75
Mission -Kocide weekly	-2.48
Mission - check	-1.92
NePlus -Kocide weekly	-2.11
NePlus - check	-1.98


8.0
6.0
4.0
2.0
0.0
LOG CELLS PER GRAM
30
40
50
60
7
DAYS AFTER JAN 1, 1983
total bacteria
INA Bacteria
-9C ice nuclei
-5C ice nuclei


8.0
6.0
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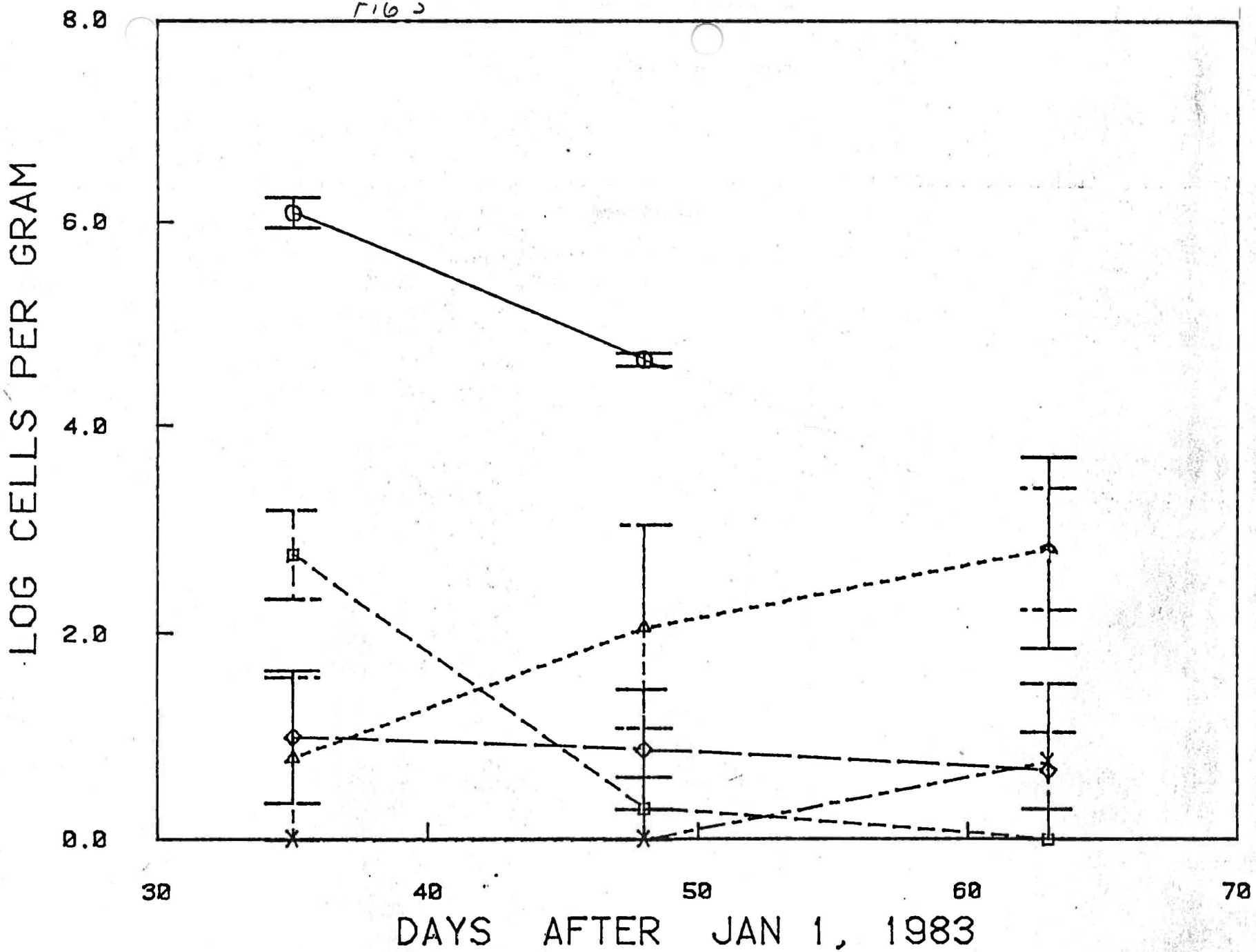
LOG CELLS PER GRAM
30
40
50
60
70
DAYS AFTER JAN 1, 1983

FIG 3


8.0
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LOG CELLS PER GRAM
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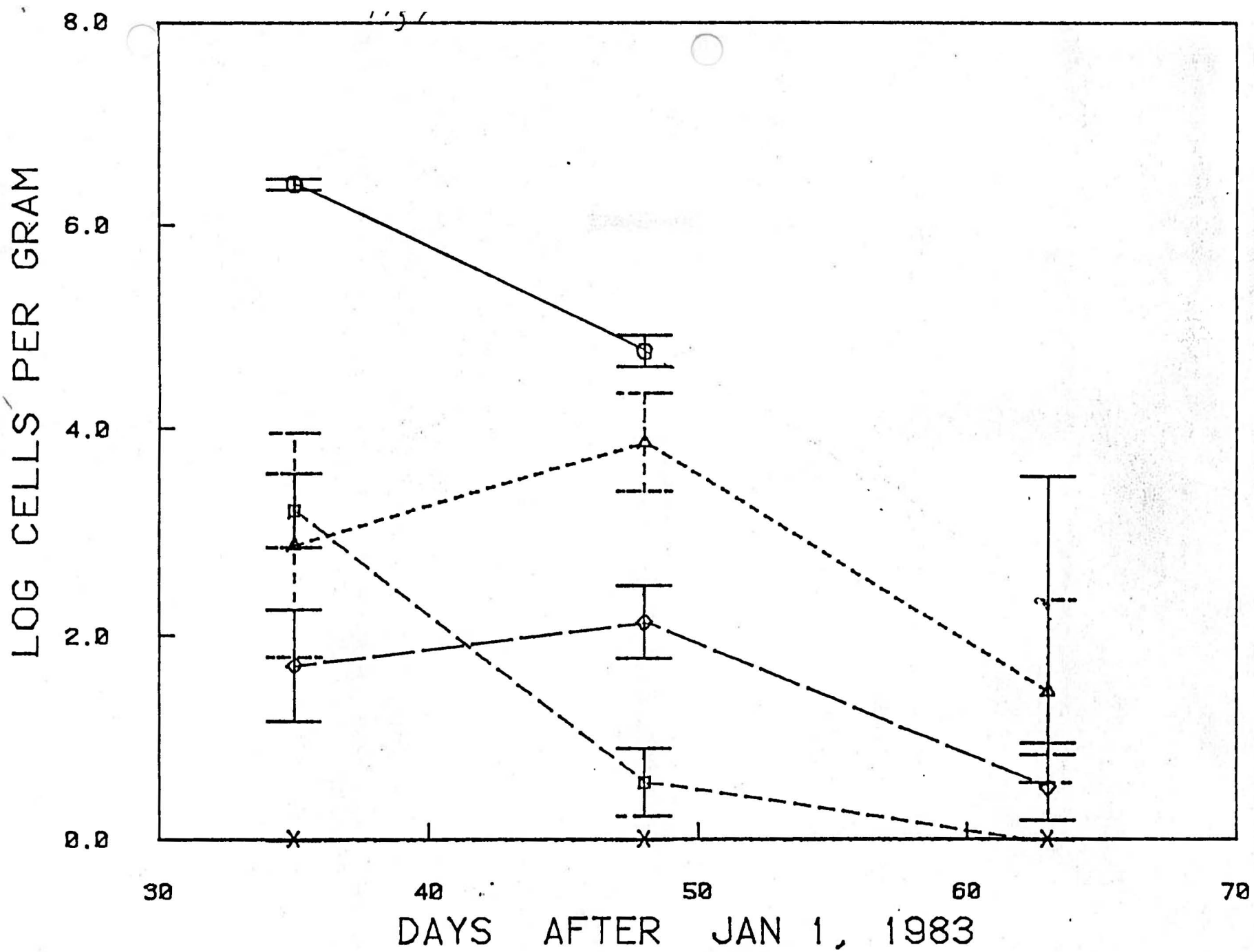
DAYS AFTER JAN 1, 1983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LOG CELLS PER GRAM
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DAYS AFTER JAN 1, 1983

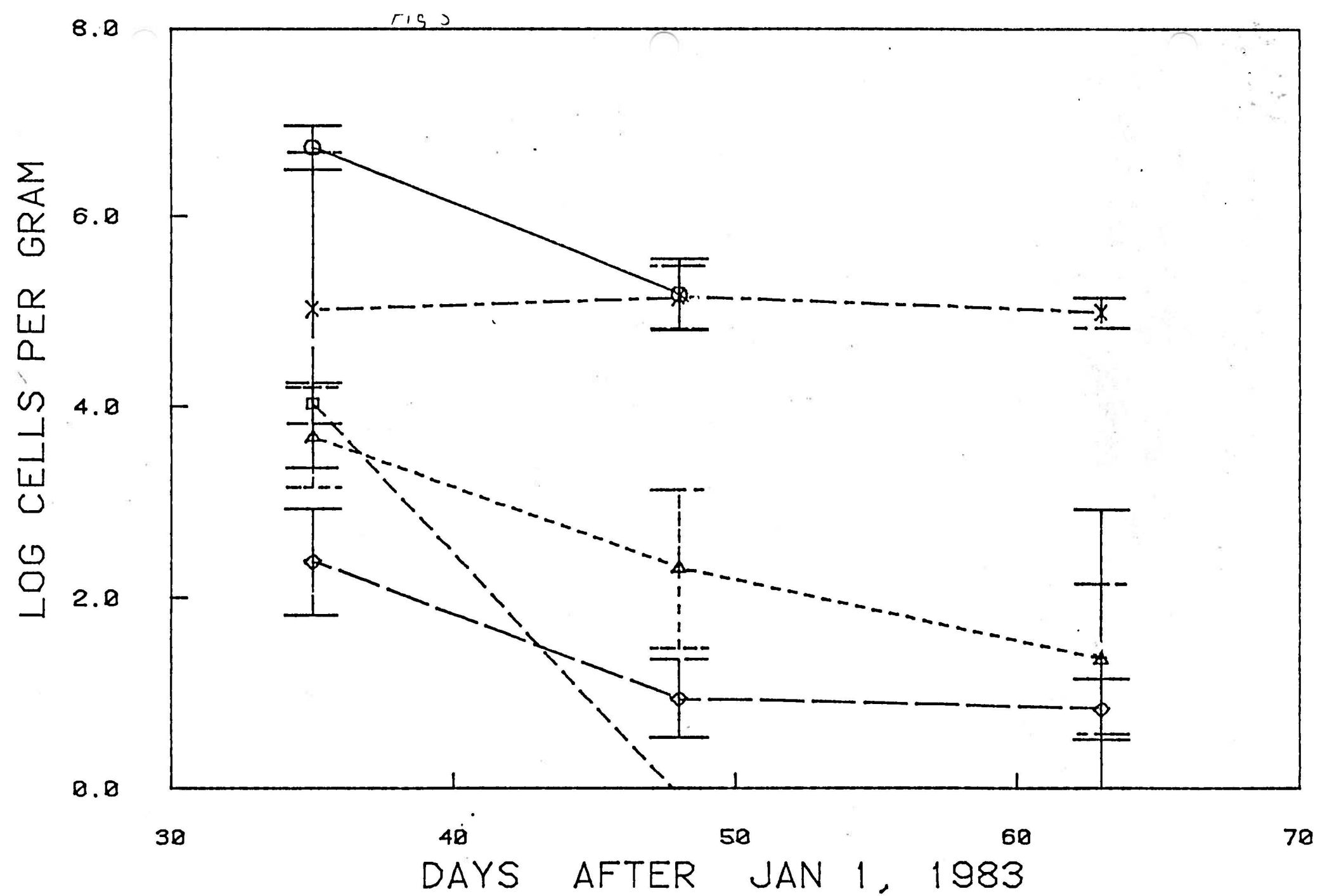
FIG 5
8.0
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4.0
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LOG CELLS PER GRAM
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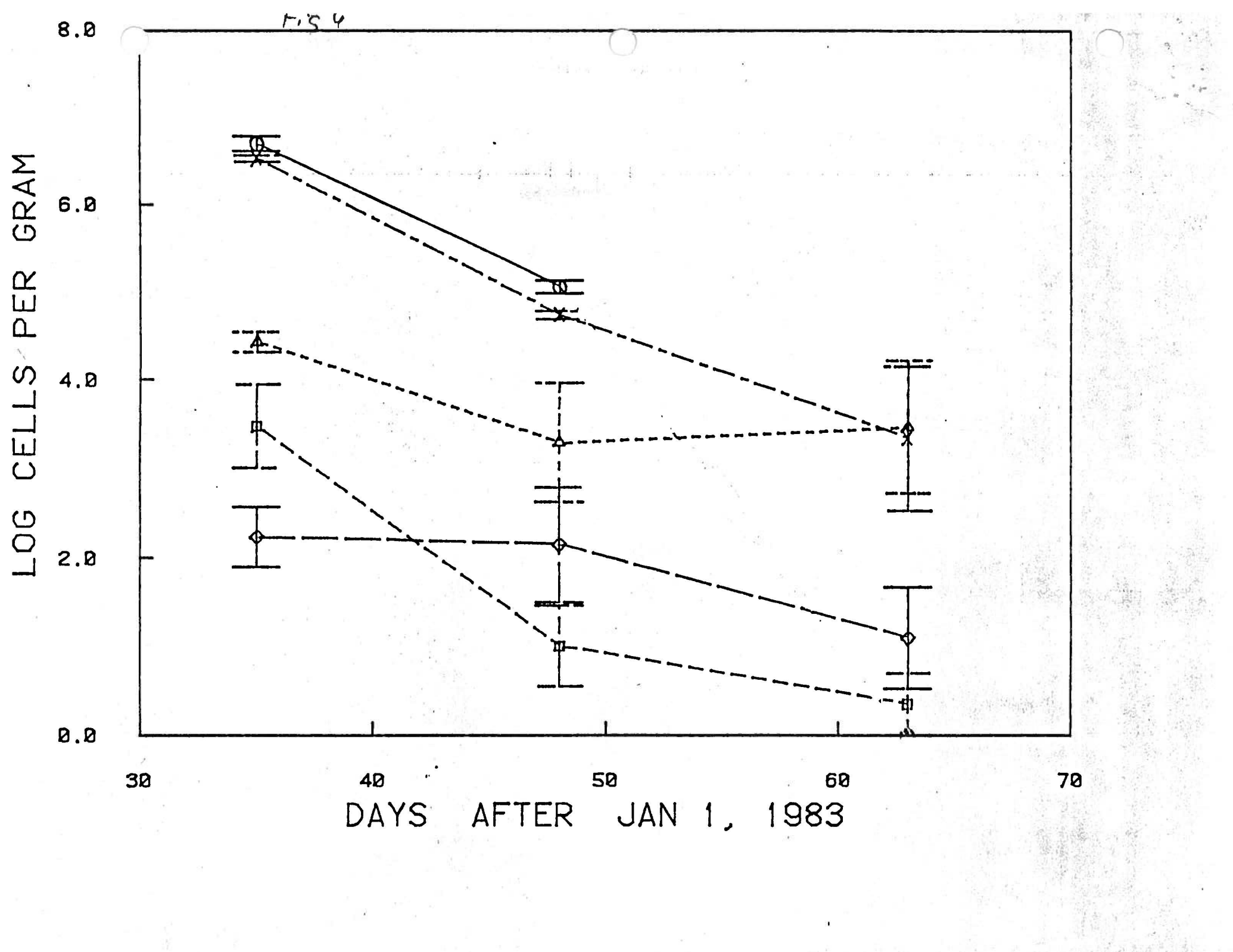
50
60
70
DAYS AFTER JAN 1, 1983


F.S 4
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LOG CELLS PER GRAM
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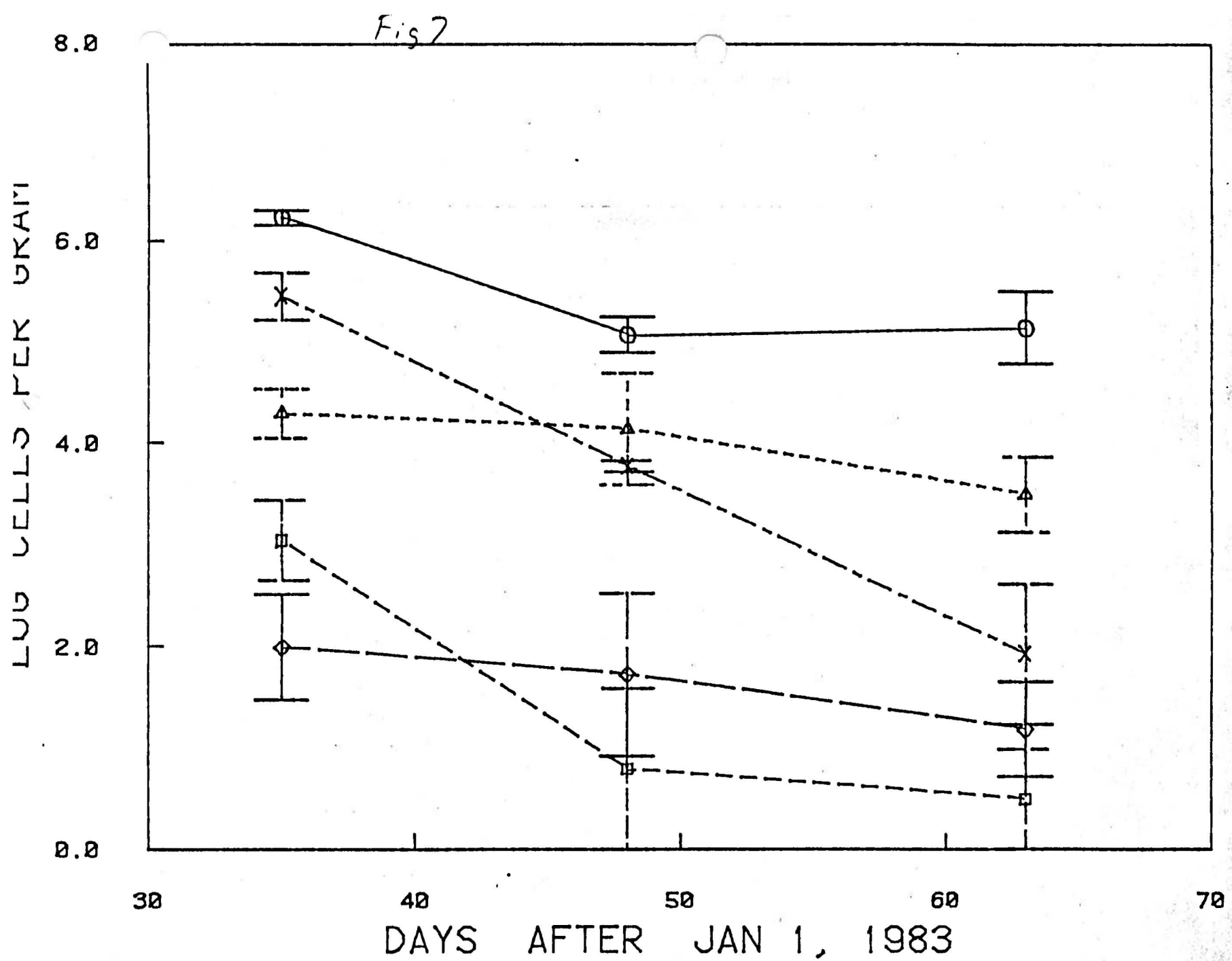
DAYS AFTER JAN 1, 1983

Fig 7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LOG CELLS PER GRAM
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70
DAYS AFTER JAN 1, 1983

Fig 8

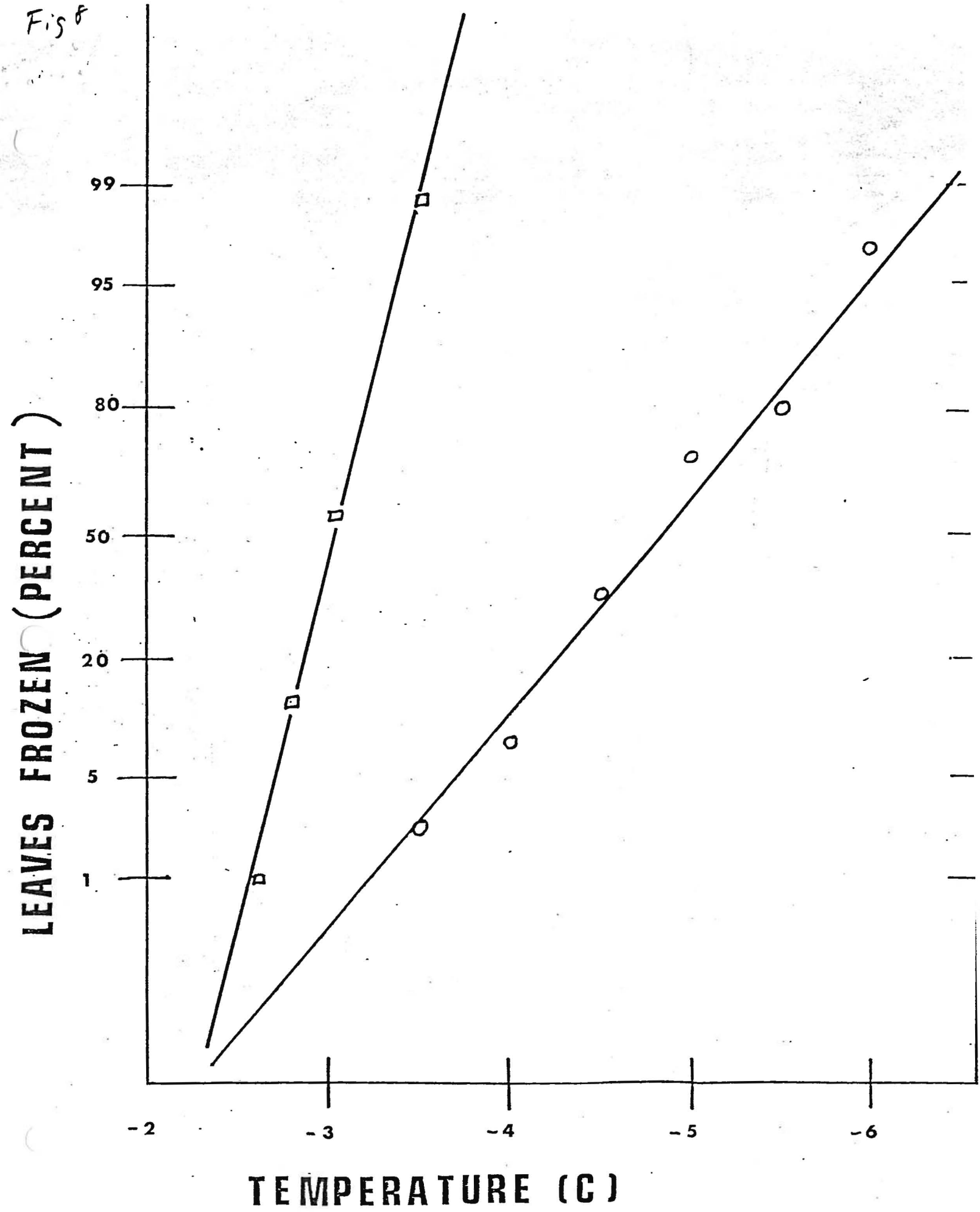
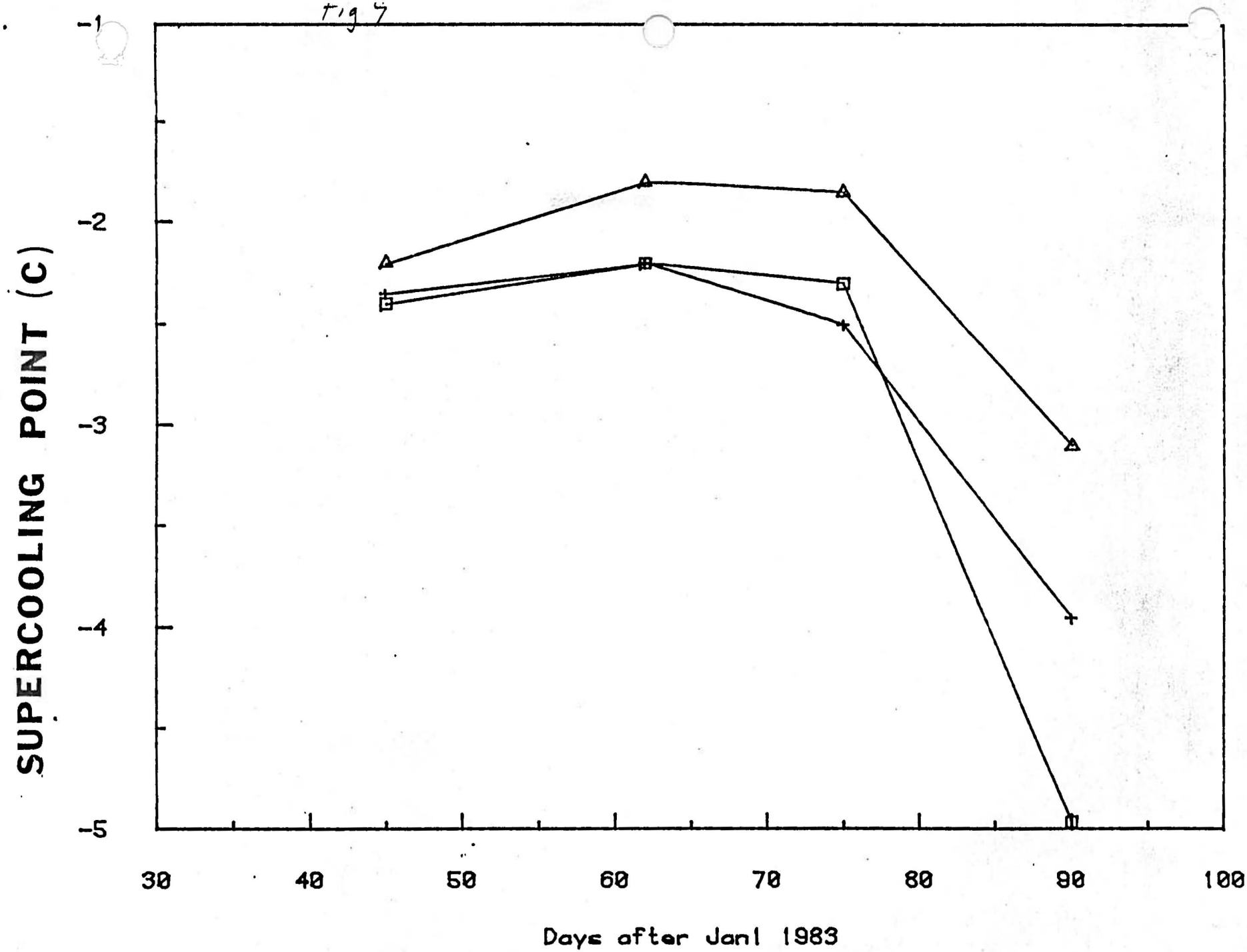


Fig 7


SUPERCOOLING POINT (C)
-1
-2
-3
-4
-5
30
40
50
60
70
80
90
100
Days after Jan1 1983