RESULTS

The different methods of preservation and storage, represented by experimental class numbers, are given in Table 1. Infections with Helicosporidium were recognized in giemsa stained smears of larvae by the presence of many spores and developmental stages of the pathogen as shown in Figures 9, 10, 11 and 12. Examination of giemsa stained slides of all control groups indicated that no Helicosporidium infection was present in the stock Aedes aegypti colony (Figure 14). Therefore, control data will be considered further only in regard to mortality.

The percentages of infection achieved in A. aegypti larvae when fresh Helicosporidium spores were used at various dosages as inoculum are shown in Table 2. The IC_{50} of fresh spores estimated from the dose-response curve constructed with these data (Figure 1), was 6×10^3 spores/ml. This served as a standard for comparison with the transmission achieved in the experimental classes.

Preliminary tests of the results of preservation and storage on infectivity of Helicosporidium spores were conducted with widely ranging dosages for reasons explained above. The results of these preliminary tests (Table 3), conducted after four weeks of storage, indicated that the infectivity of Helicosporidium spores was retained in every class except class XI. However, transmission rates achieved with spores of classes V, IX and X were very low, indicating that preservation and storage methods represented by these class numbers were of questionable practical value. Therefore, no further consideration was given classes V, IX, X and XI. Where possible, IC_{50}'s were estimated from curves constructed from data in Table 3 (Figures 2 a-g). The narrower ranges of spore concentrations within which more precise IC_{50}'s were sought in the definitive tests that followed were also derived from these curves.

The results of definitive tests conducted after eight weeks of storage are given in Table 4. These data were used to construct dose-response curves (Figures 3 a-f) for each class of the experiment, and the IC_{50}'s in Table 4 were estimated from these. It will be noted that the infectivity of spores in class IV decreased dramatically from that found after four weeks of storage. Even at the highest dosage used, 10^6 spores/ml, there was less than 25 percent transmission. Storage by the method represented by class IV was considered to be of questionable practical value. Therefore, class IV was not considered in subsequent tests.

The results of infectivity tests conducted after 12 weeks of storage are given in Table 5. Spores in class I produced 100 percent transmission at all dosages used, making it impossible to construct a dose-response curve for that class. Dose-response curves were constructed with data from class III, II-1, VI, II-2 and VII (Figures 4 a-e), and IC_{50}'s estimated from these are given in Table 5.

The results of infectivity tests conducted after 16 weeks of storage are given in Table 6. Dose-response curves were constructed with these data (Figures 5 a-f) and IC_{50}'s estimated from these are given in Table 6.

These data indicate a direct relationship between dose (spores/ml) and percent transmission of Helicosporidium to Aedes aegypti. Evidently, preservation and storage methods involving the desiccation of Helicosporidium spores are severely detrimental to infectivity of the spores and are contraindicated. Preservation and storage methods involving freezing of Helicosporidium spores, whether in intact larvae or as spore suspensions in the protectants used, are favourable to the retention of infectivity of the spores. It was expected that the IC_{50}'s of spores preserved and stored by favourable methods would either remain constant with duration of storage or show a predictable rate of decrease. However, the effects of duration of storage on the IC_{50}'s is not clear from these data (Table 7 and Figure 6), and possible explanations of this are discussed below. It appears safe to conclude, however, that preservation and storage of Helicosporidium spores by methods represented by classes I, III, II-1, VI, II-2 and VII (Table 1) is possible for up to four months with little loss of infectivity. These methods will be of great value to researchers working with this species. It appears probable that storage by these methods will also be possible for sufficient durations to be of industrial interest.

The percent mortality of A. aegypti larvae which were exposed to fresh Helicosporidium spores and spores preserved for 4, 8, 12 and 16 weeks are given in Tables 8, 9, 10, 11 and 12 respectively. The percent mortality for the respective control groups are also given. The mean percent mortality of the control was 2.25. Mortality of exposed larvae was bimodal, the first peak usually occurring on the second day after exposure, with a much larger second peak occurring during the late fourth stage or the pupal stage.

A correlation between IC_{50} and mean temperature changes was suggested by Table 13, Figures 6 and 7. The IC_{50}'s of the four Helicosporidium spore suspension groups followed for 16 weeks showed fluctuations which were directly related to mean temperature changes of the pre-exposure incubation days of the experimental larvae. The effects of temperature of incubation days on the IC_{50}'s of the two intact larvae groups followed for 16 weeks was partially contradictory, but the weight of evidence suggests and logic supports the existence of a strong direct correlation between temperature of incubation days and the IC_{50}'s. This is further discussed below.

Table 1. Class numbers of different methods of treatment and storage.

Material	Treatment	Protectant	Storage	Class no.
Intact larvae	Freezing in LN_2	-	Liquid Nitrogen (LN_2)	I
			REVCO freezer	III
	Vacuum drying	-	REVCO freezer	IV
			Room temperature	IX
	Lyophilization	-	REVCO freezer	V
			Room temperature	X
Spore suspension	Freezing in LN_2	E_1	LN_2	II-1
			REVCO freezer	VI
		E_2	LN_2	II-2
			REVCO freezer	VII
	Lyophilization	E_2	Room temperature	XI

Table 2. Percentages of infection of 48 hr old Aedes aegypti larvae exposed to fresh Helicosporidium spores at various dosages and the IC_{50} estimated from a graph of the data.

Dosages (Spores/ml)	2×10^3	10^4	2×10^4	3×10^4	4×10^4	5×10^4	6×10^4	7×10^4	8×10^4	10^5
Percent infection	32	58	80	82	82	88	90	90	90	98

Estimated $IC_{50} = 6 \times 10^3$ spores/ml.

Table 3. Percentages of infection of 48 hr old _Aedes aegypti_ larvae exposed to various dosages of _Helicosporidium_ spores after 4 weeks of storage and the IC_{50}'s estimated from graphs of the data.

Class no.	Dosages (spores/ml)				IC_{50} (Spores/ml)
	5×10^2	5×10^3	5×10^4	5×10^5	
I	8	62	92	100	3.5×10^3
III	12	64	100	100	3×10^3
IV	-	4	10	68	2.3×0^5
IX	2	-	2	4	$>5 \times 10^5$
V	-	-	-	6	$>5 \times 10^5$
X	-	2	-	2	$>5 \times 10^5$
II-1	2	24	92	98	1.2×10^4
VI	2	12	88	100	1.5×10^4
II-2	6	20	88	96	1.4×10^4
VII	10	26	72	100	1.4×10^4

Table 4. Percentages of infection of 48 hr old <u>Aedes aegypti</u> larvae exposed to various dosages of <u>Helicosporidium</u> spores after 8 weeks of storage and the IC_{50}'s estimated from graphs of the data.

Class no.	Dosages (spores/ml)							IC_{50} (Spores/ml)
	10^3	5×10^3	10^4	5×10^4	10^5	5×10^5	10^6	
I	26	32	48	90	-	-	-	10^4
III	20	54	58	100	-	-	-	4.5×10^3
IV	-	-	-	-	2	14	22	$>10^6$
II-1	-	48	66	98	100	-	-	5.6×10^3
VI	-	44	62	96	100	-	-	6.2×10^3
II-2	-	48	64	100	100	-	-	5.4×10^3
VII	-	72	82	100	100	-	-	0.9×10^3

Table 5. Percentages of infection of 48 hr old <u>Aedes aegypti</u> larvae exposed to various dosages of <u>Helicosporidium</u> spores after 12 weeks of storage and the IC_{50}'s estimated from graphs of the data.

Class no.	Dosages (spores/ml)						IC_{50} (Spores/ml)
	10^3	5×10^3	10^4	5×10^4	10^5	5×10^5	
I	-	-	100	100	100	100	$< 10^4$
III	22	82	86	100	-	-	2.5×10^3
II-1	-	28	38	88	96	-	1.4×10^4
VI	-	36	48	84	96	-	1.1×10^4
II-2	-	34	80	86	94	-	10^4
VII	-	14	28	78	92	-	2×10^4

Table 6. Percentages of infection of 48 hr old <u>Aedes aegypti</u> larvae exposed to various dosages of <u>Helicosporidium</u> spores after 16 weeks of storage and the IC_{50}'s estimated from graphs of the data.

Class no.	Dosages (spores/ml)					IC_{50} (Spores/ml)
	10^3	5×10^3	10^4	5×10^4	10^5	
I	28	76	92	98	-	2.1×10^3
III	58	92	92	100	-	0.6×10^3
II-1	-	50	54	100	98	6.4×10^3
VI	-	50	72	100	100	5×10^3
II-2	-	52	90	100	100	4.5×10^3
VII	-	64	86	100	100	2×10^3

Table 7. Relationship between IC_{50} (Spores/ml) and duration of storage.

Class no.	Duration of Storage			
	4 weeks	8 weeks	12 weeks	16 weeks
I	3.5×10^3	10^4	$< 10^4$	2.1×10^3
III	3×10^3	4.5×10^3	2.5×10^3	0.6×10^3
IV	2.3×10^5	$>10^6$	-	-
II-1	1.2×10^4	5.6×10^3	1.4×10^4	6.4×10^3
VI	1.5×10^4	6.2×10^3	1.1×10^4	5×10^3
II-2	1.4×10^4	5.4×10^3	10^4	4.5×10^3
VII	1.4×10^4	0.9×10^3	2×10^4	2×10^3

Table 8. Percentages of mortality of *Aedes aegypti* larvae which were exposed to various dosages of fresh *Helicosporidium* spores at 48 hr of age.

Dosages (Spores/ml)	2×10^3	10^4	2×10^4	3×10^4	4×10^4	5×10^4	6×10^4	7×10^4	8×10^4	10^5
Percent Mortality	4.75	5	1.5	1.75	1.25	1	4.5	1.75	0.75	5

Control group mortality = 1.25 percent.

Table 9. Percentages of mortality of *Aedes aegypti* larvae which were exposed to *Helicosporidium* spores after 4 weeks of storage.

Class no.	Dosages (Spores/ml)			
	5×10^2	5×10^3	5×10^4	5×10^5
I	11.25	24	12.75	28.5
III	12	20.75	12.75	25
IV	6	8.5	11.75	4.25
IX	10.5	16	15	5.5
V	13.25	20.5	9.5	6.75
X	17.75	26	13.25	6.75
II-1	2.5	2.75	7.25	3.5
VI	6.25	6.25	17	34.75
II-2	5.5	6.5	8.5	22
VII	21.5	22	31.25	33
XI	15.75	13	12.5	1.75

Mortality in controls for Classes I, III, IV, V, IX and X = 0 percent

Mortality in controls for Classes II-1, II-2, VI, VII and XI = 10 percent

Table 10. Percentages of mortality of *Aedes aegypti* larvae which were exposed to *Helicosporidium* spores after 8 weeks of storage.

Class no.	Dosages (Spores/ml)						
	10^3	5×10^3	10^4	5×10^4	10^5	5×10^5	10^6
I	5.25	3.5	6	13	-	-	-
III	7.25	10.5	6	6.5	-	-	-
IV	-	-	-	5.5	5.5	3.25	3
II-1	-	24.5	20.25	41	61	-	-
VI	-	10	7.5	18.25	34	-	-
II-2	-	10	7.75	23.25	35	-	-
VII	-	10.5	10	20.5	53	-	-

Mortality in controls for Classes I, III and IV = 0.75 percent

Mortality in controls for Classes II-1, II-2, VI and VII = 1 percent

Table 11. Percentages of mortality of *Aedes aegypti* larvae which were exposed to *Helicosporidium* spores after 12 weeks of storage.

Class no.	Dosages (Spores/ml)					
	10^3	5×10^3	10^4	5×10^4	10^5	10^6
I	-	-	13.5	2	35.25	73.25
III	14	10.25	12	41.75	-	-
II-1	-	10.75	4	9	6	-
VI	-	5.25	15.25	12.75	9.75	-
II-2	-	14	9.25	10.75	14.75	-
VII	-	12.5	5.5	11.25	10.75	-

Mortality in controls for Classes I and III = 0 percent

Mortality in controls for Classes II-1, II-2, VI and VII = 0.25 percent

Table 12. Percentages of mortality of Aedes aegypti larvae which were exposed to Helicosporidium spores after 16 weeks of storage.

Class no.	Dosages (Spores/ml)				
	10^3	5×10^3	10^4	5×10^4	10^5
I	0.5	1	1.25	19.75	-
III	0.5	1.25	3.25	17	-
II-1	-	0.5	1.25	21.25	30.75
VI	-	-	1.25	18.25	43.5
II-2	-	1.5	1	20.75	30.75
VII	-	1	2.25	40.5	49.75

Mortality in controls for Classes I and III = 0.25 percent

Mortality in controls for Classes II-1, II-2, VI and VII = 0 percent

Table 13. Relationship between IC_{50} (Spores/ml) and mean temperature (°C) of incubation days, the 2 days before infectivity tests of preserved Helicosporidium spores, after storages of various duration (weeks).

Class no.	Storage duration (weeks)							
	4		8		12		16	
	IC_{50}	Temp (°C)	IC_{50}	Temp (°C)	IC_{50}	Temp (°C)	IC_{50}	Temp (°C)
I	3.5×10^3	25.1	10^4	24.6	$<10^4$	23.8	2.1×10^3	25.0
III	3×10^3		4.5×10^3		2.5×10^3		0.6×10^3	
II-1	1.2×10^4	25.4	5.6×10^3	25.2	1.4×10^4	26.6	6.4×10^3	25.4
VI	1.5×10^4		6.2×10^3		1.1×10^4		5×10^3	
II-2	1.4×10^4		5.4×10^3		10^4		4.5×10^3	
VII	1.4×10^4		0.9×10^3		2×10^4		2×10^3	

IC_{50} of fresh Helicosporidium spores = 6×10^3 spores/ml. Mean temperature (°C) of incubation days before infectivity test of fresh Helicosporidium spores = 26.04 °C.

Fig I. Dose-response curve of fresh spores.

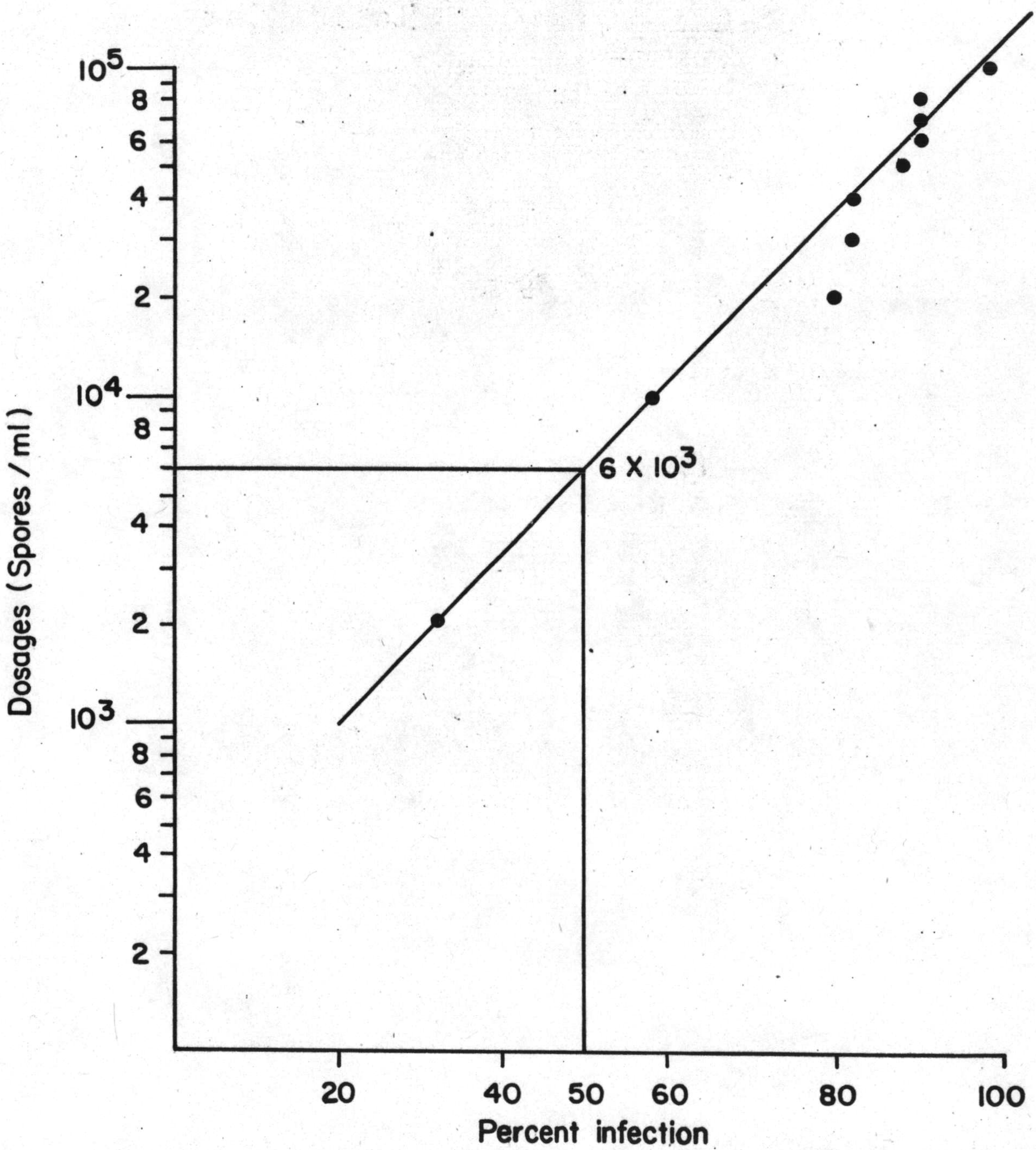


Fig. 2a. Dose-response curve of class I, 4 weeks after storage.

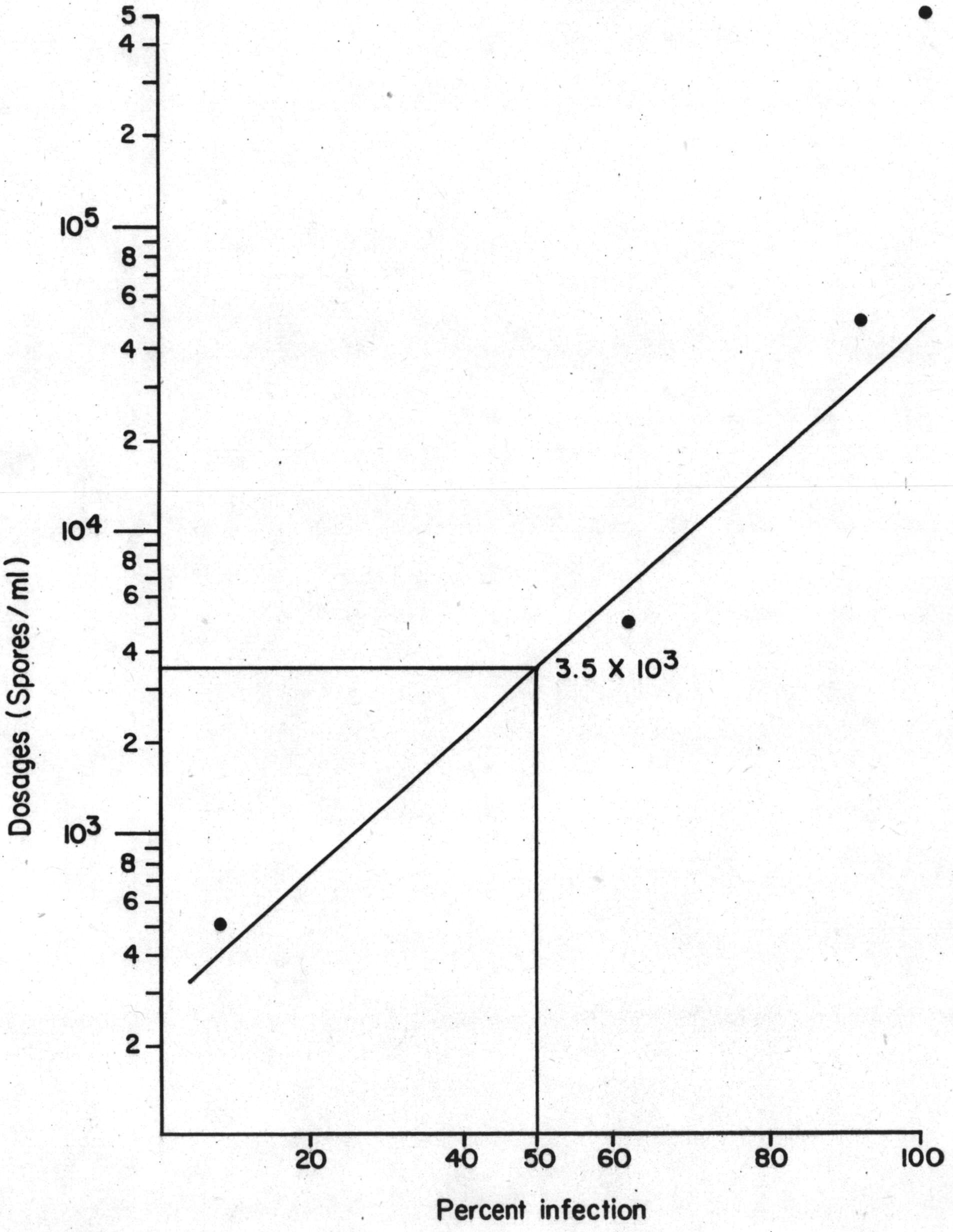


Fig 2b. Dose−response curve of class III, 4 weeks after storage.

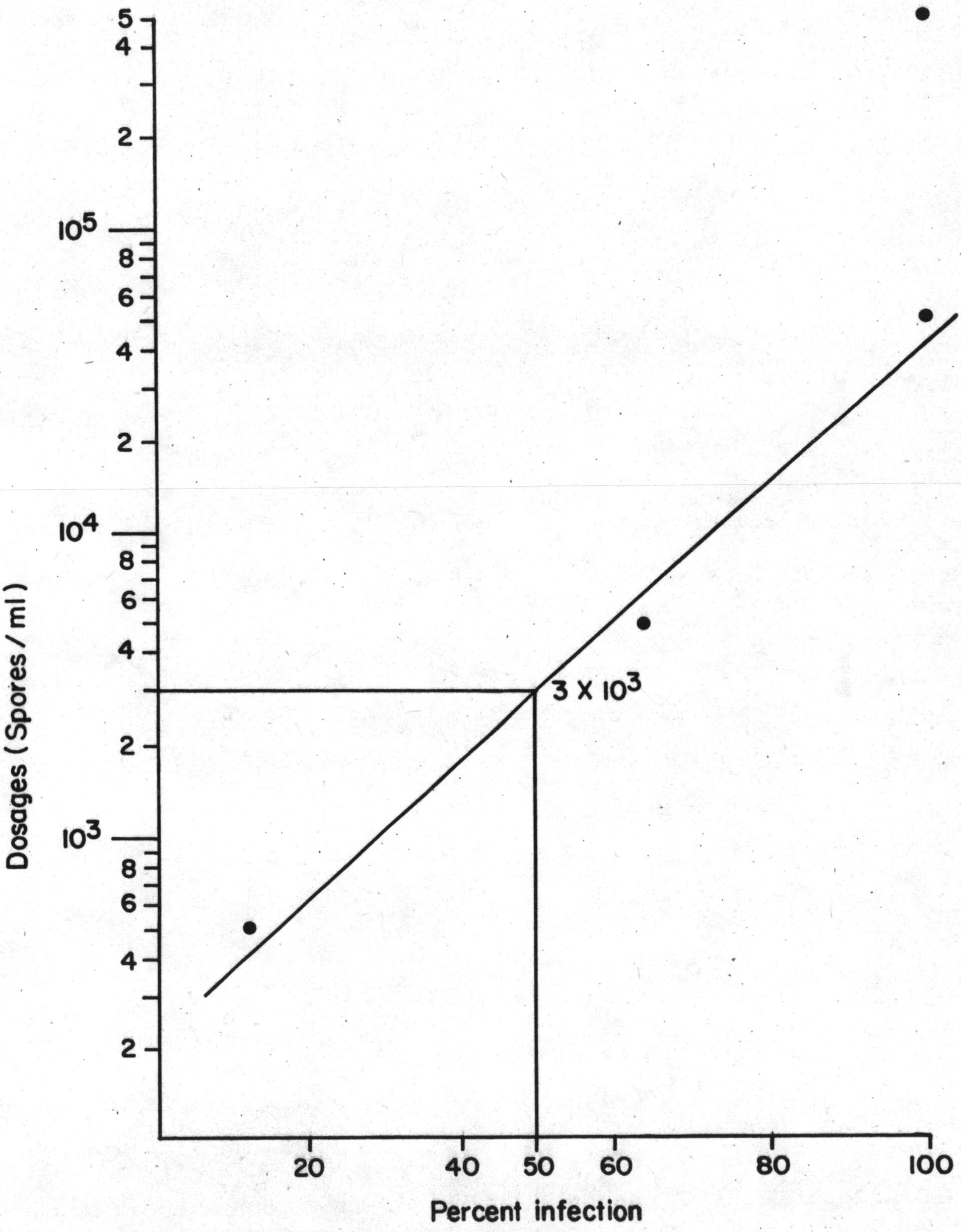


Fig. 2c. Dose-response curve of class IV, 4 weeks after storage.

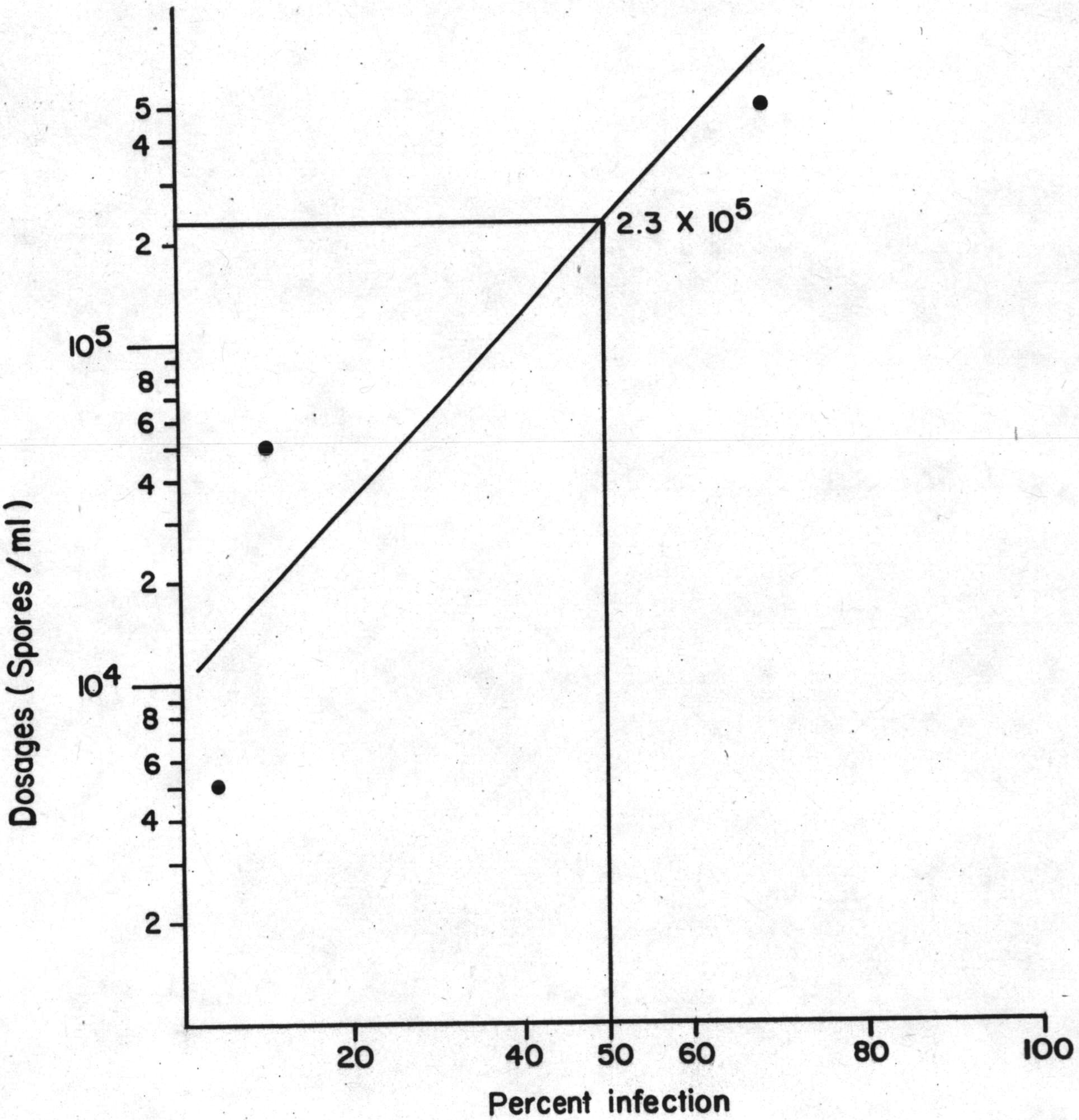


Fig. 2d. Dose-response curve of class II-I, 4 weeks after storage.

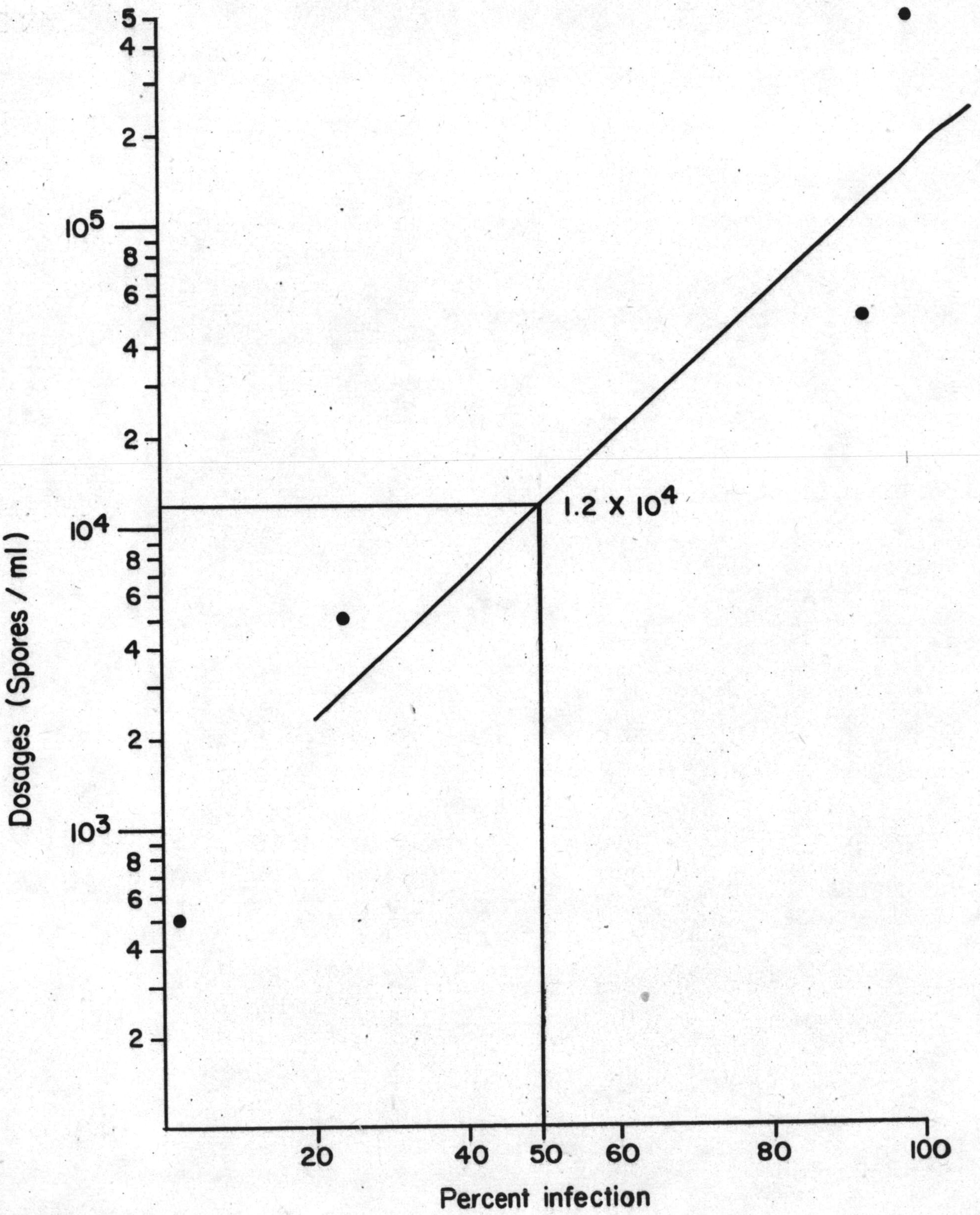


Fig 2e. Dose-response curve of class VI, 4 weeks after storage.

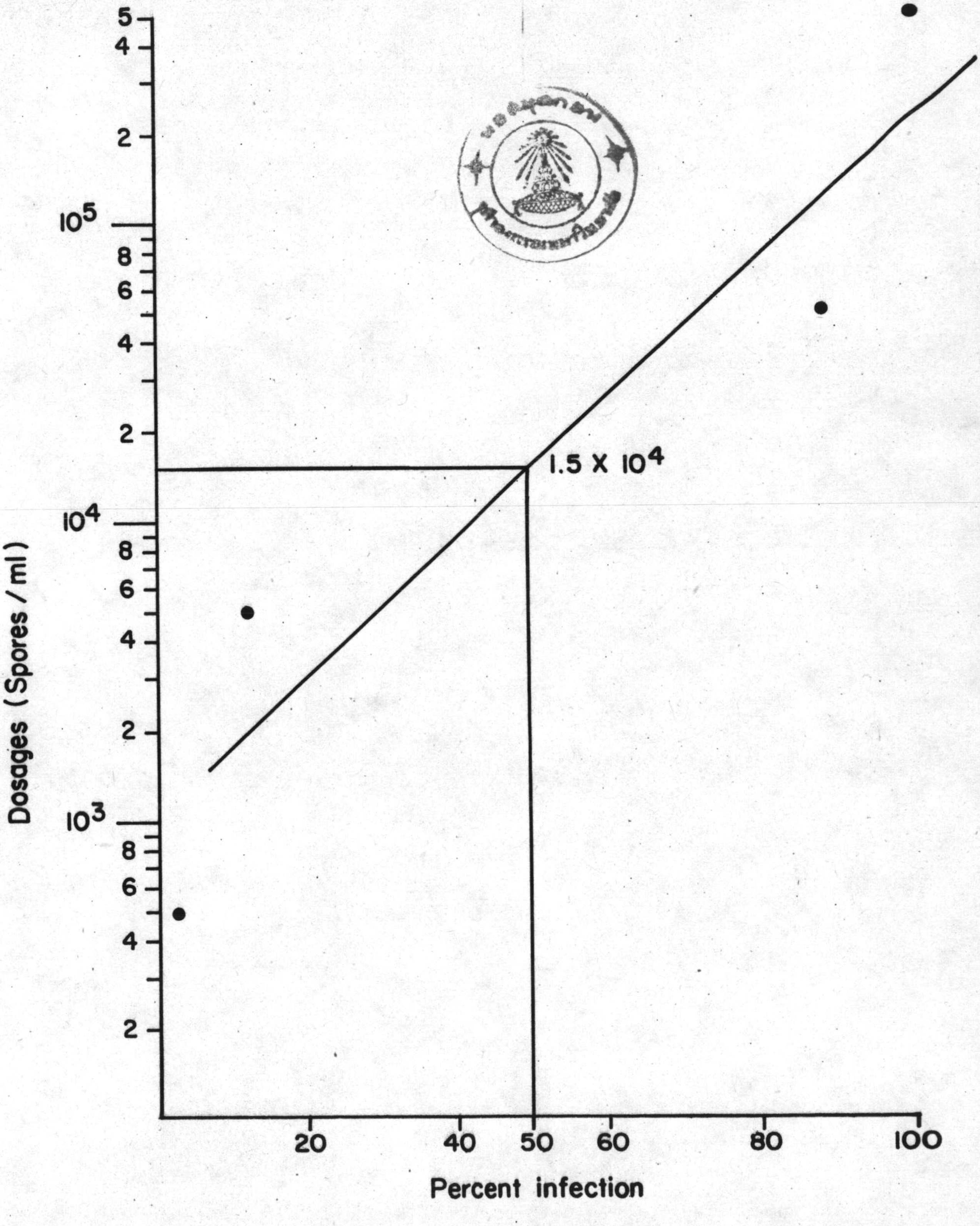


Fig 2f. Dose-response curve of class II-2, 4 weeks after storage.

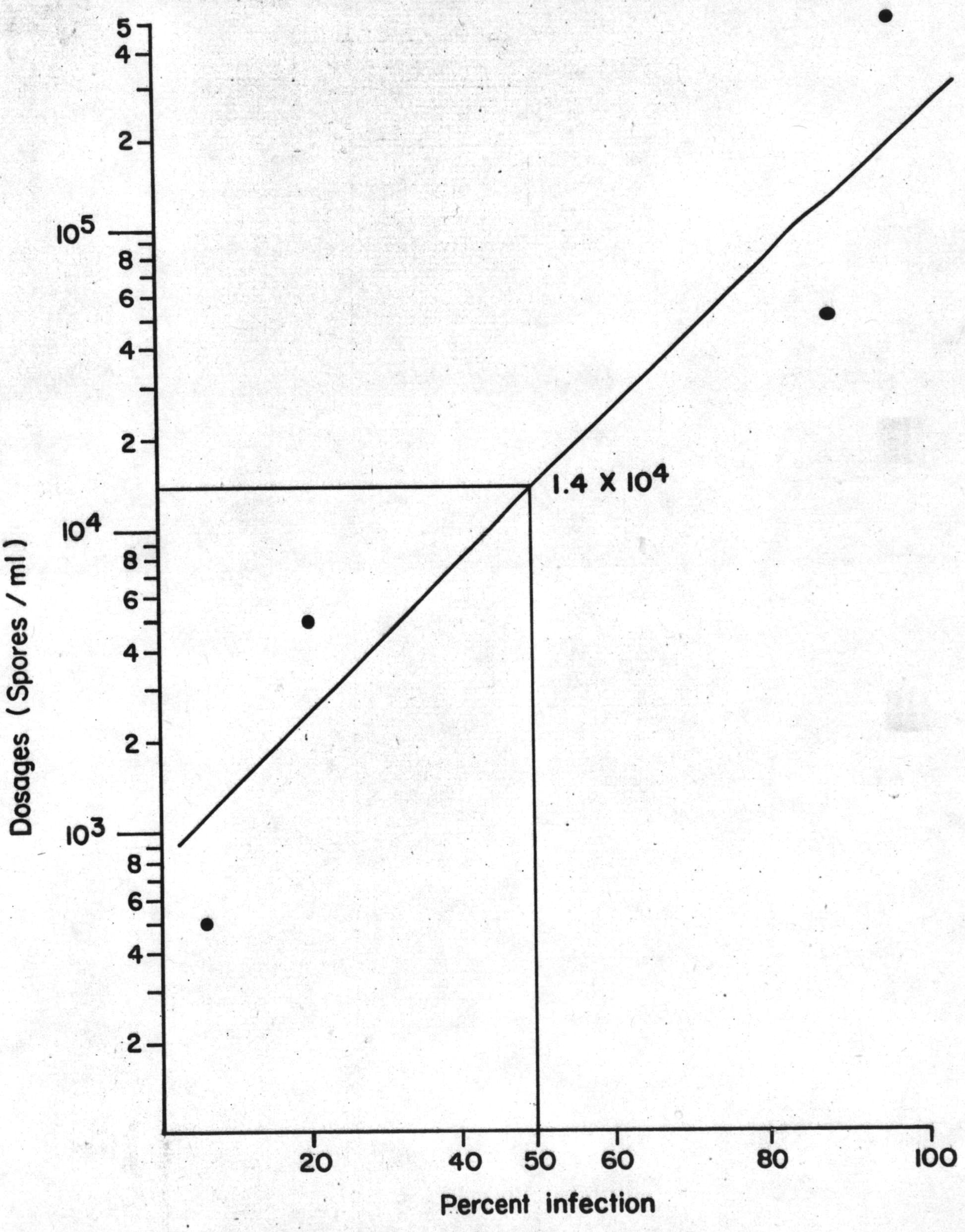


Fig 2g. Dose-response curve of class VII, 4 weeks after storage.

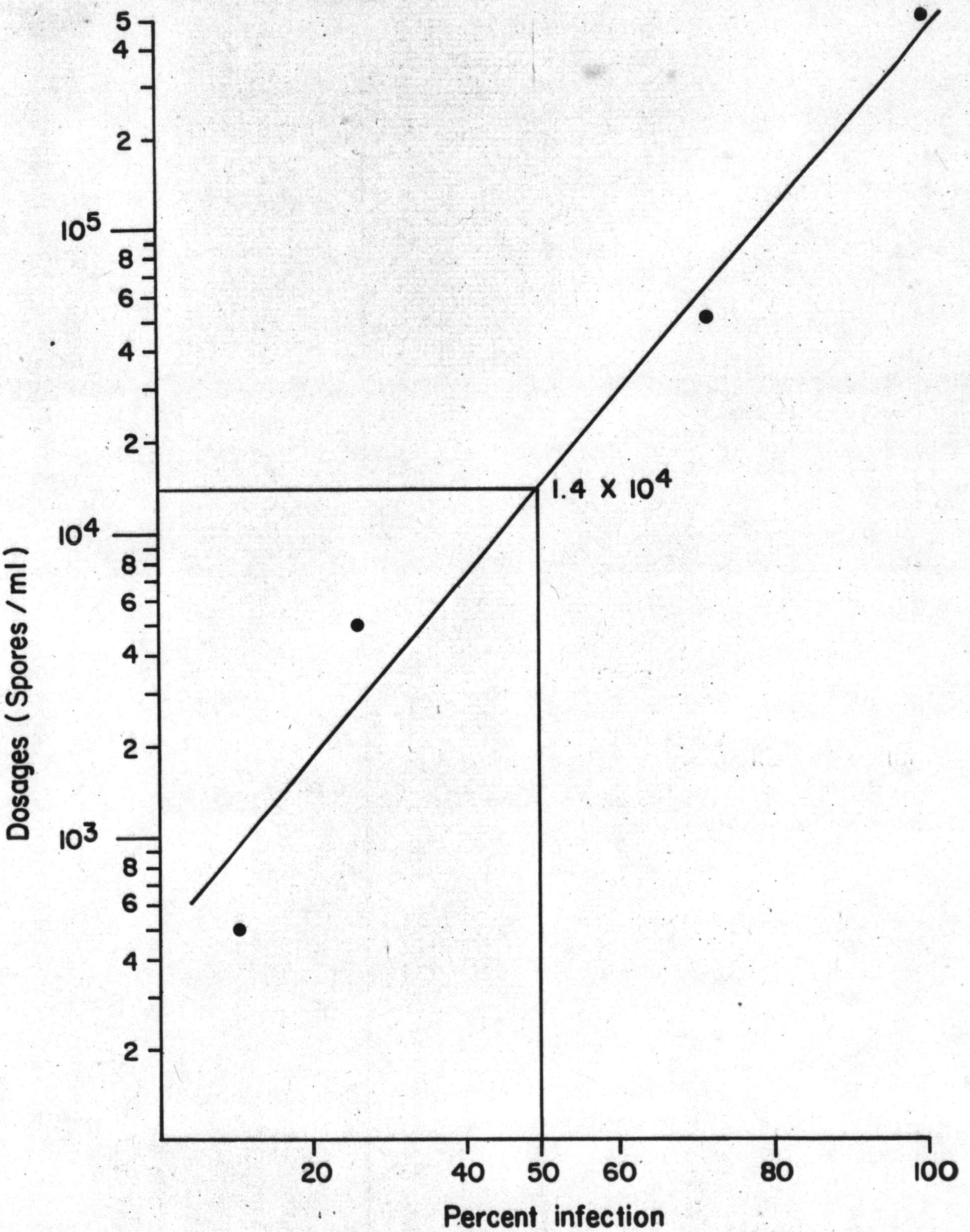


Fig 3a. Dose–response curve of class I, 8 weeks after storage.

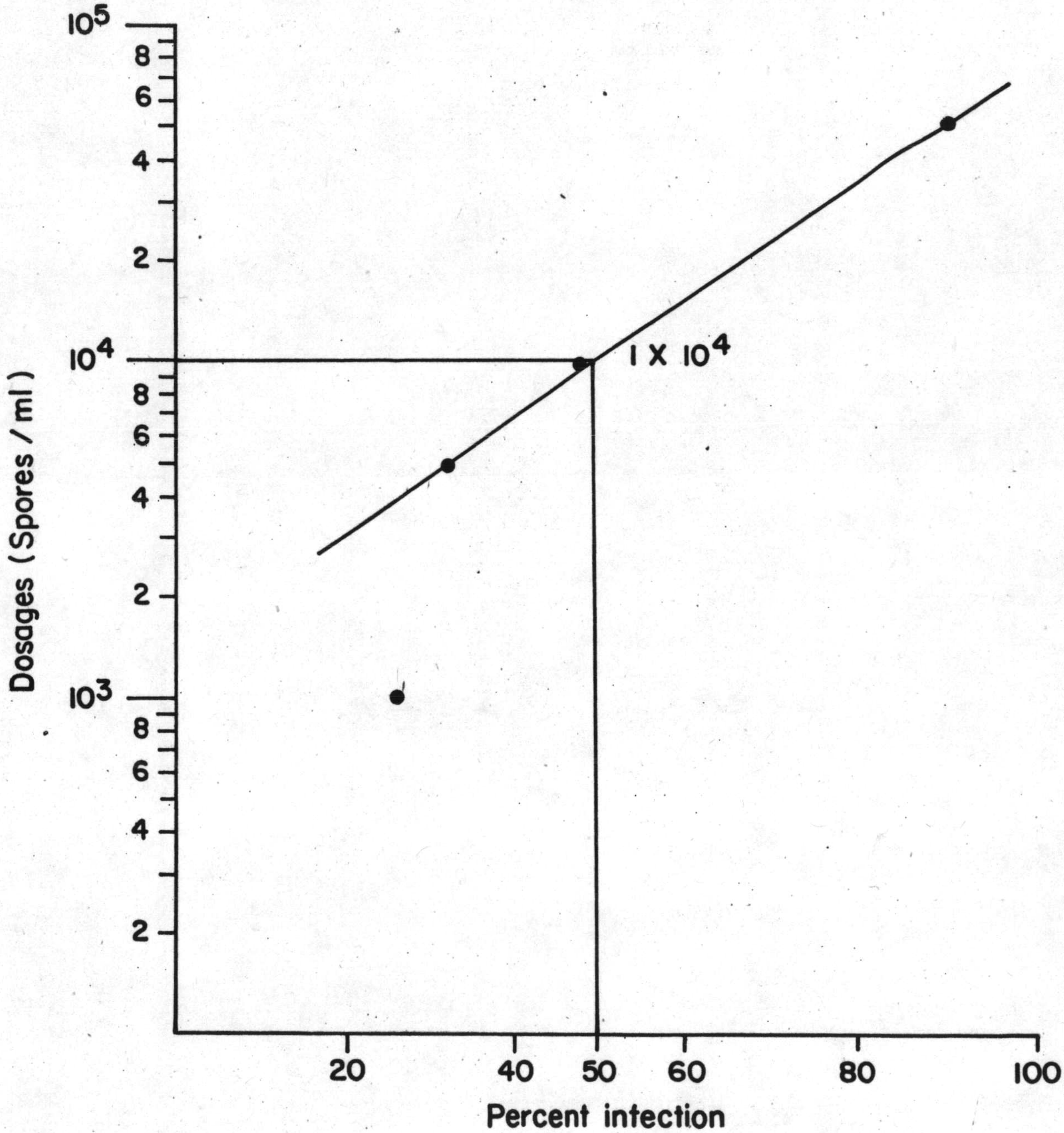


Fig 3b. Dose-response curve of class III, 8 weeks after storage.

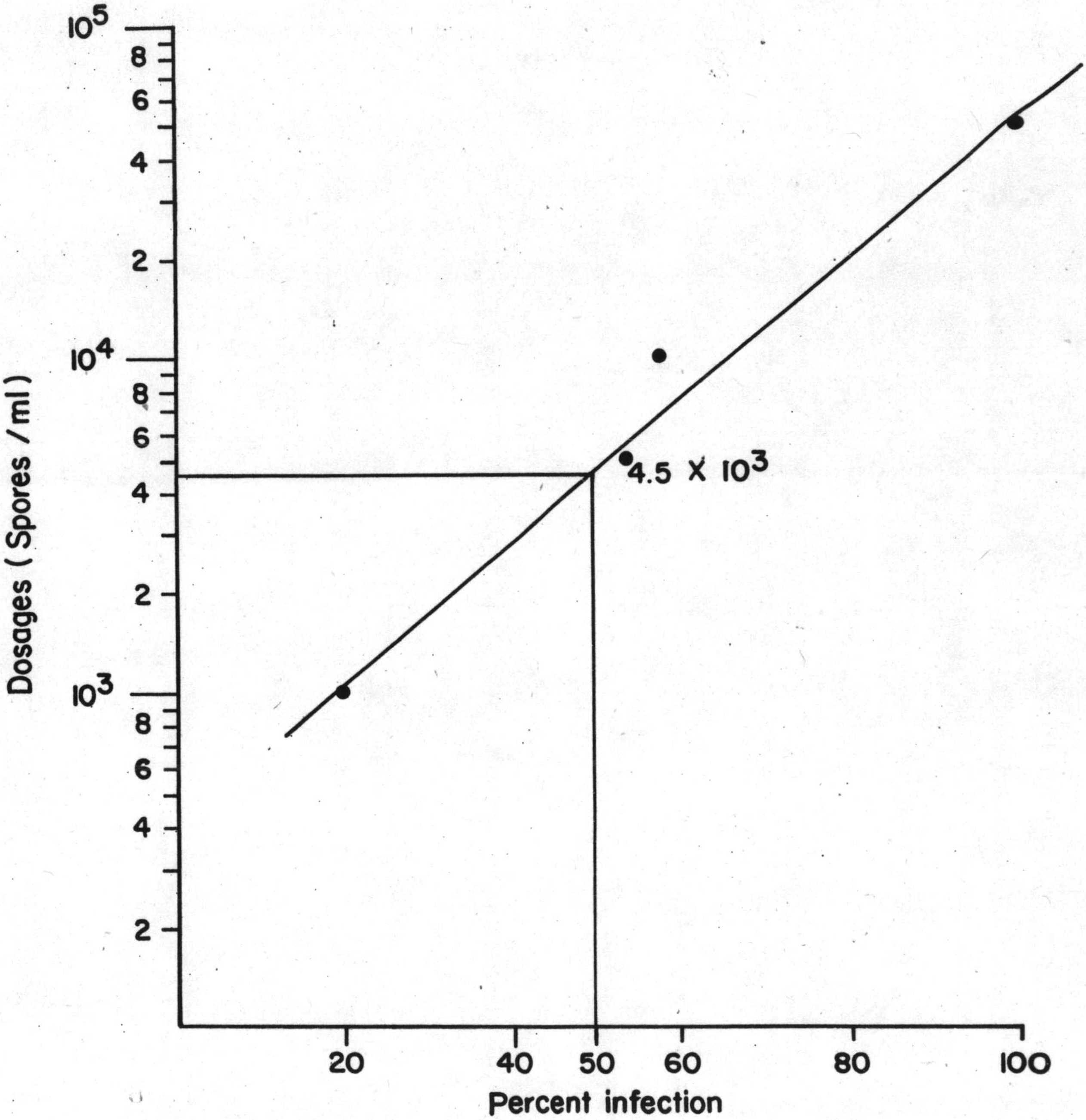


Fig 3c. Dose-response curve of class II-I, 8 weeks after storage.

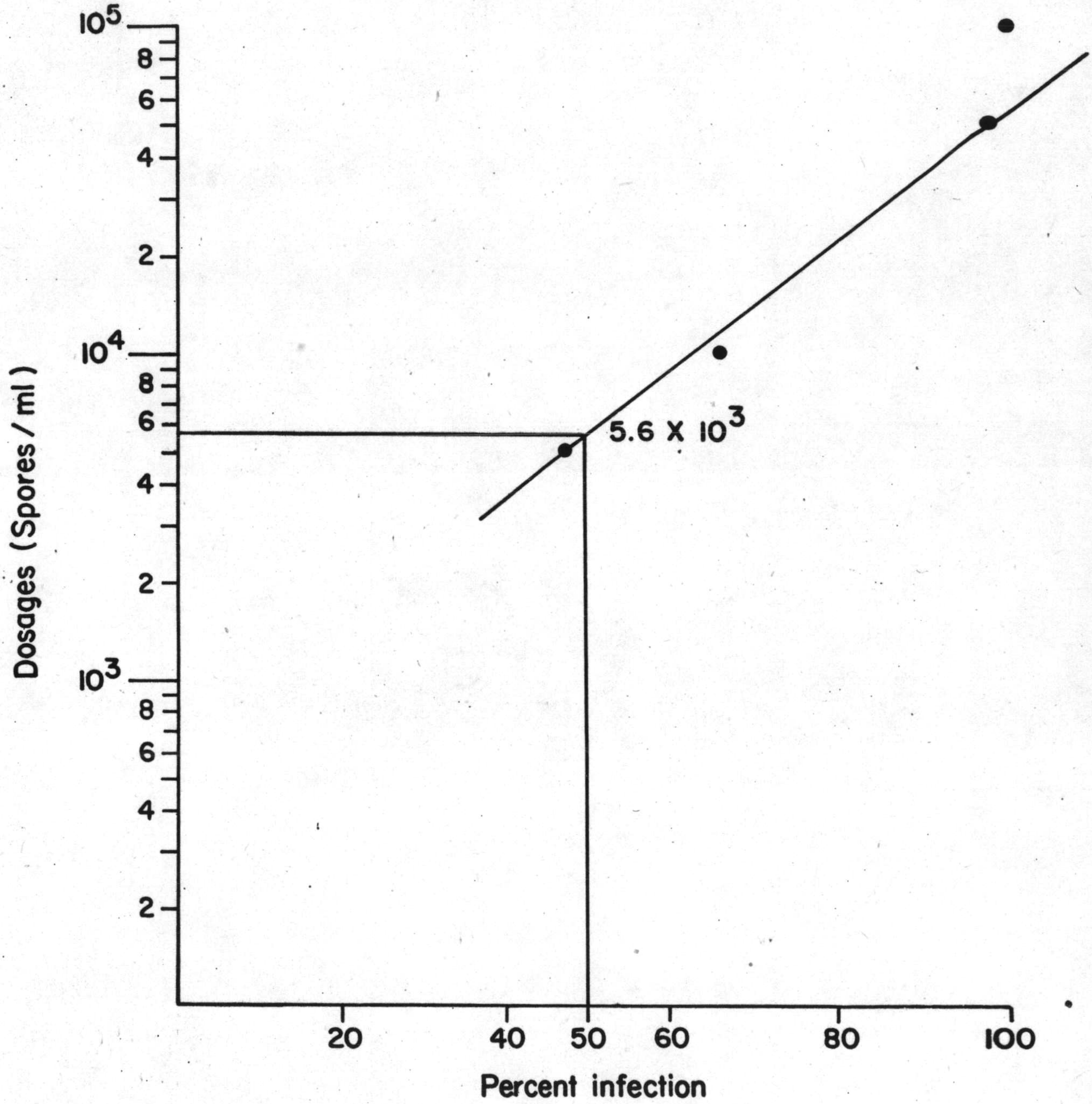


Fig 3d. Dose-response curve of class VI, 8 weeks after storage.

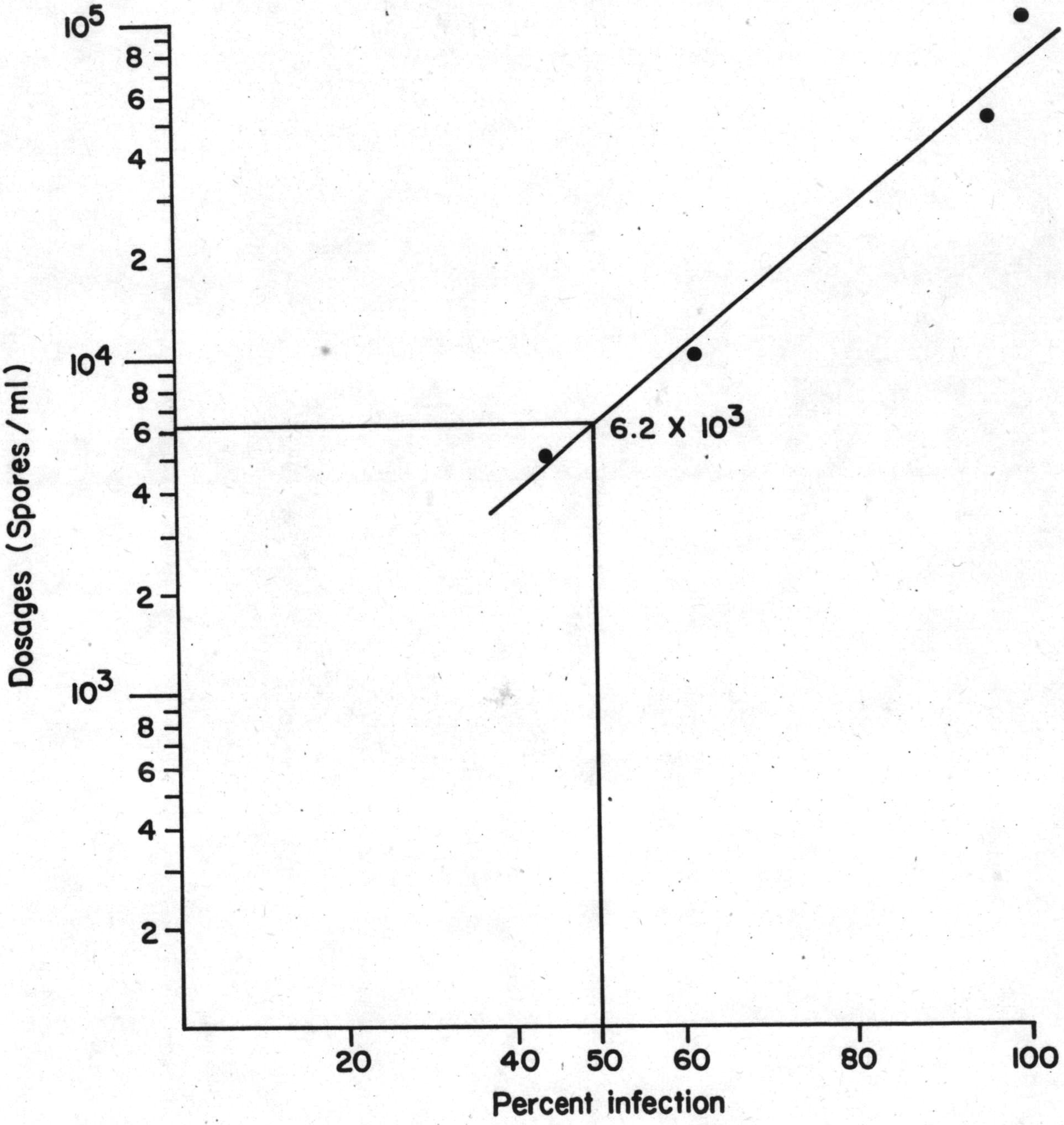


Fig 3e. Dose-response curve of class II-2, 8 weeks after storage.

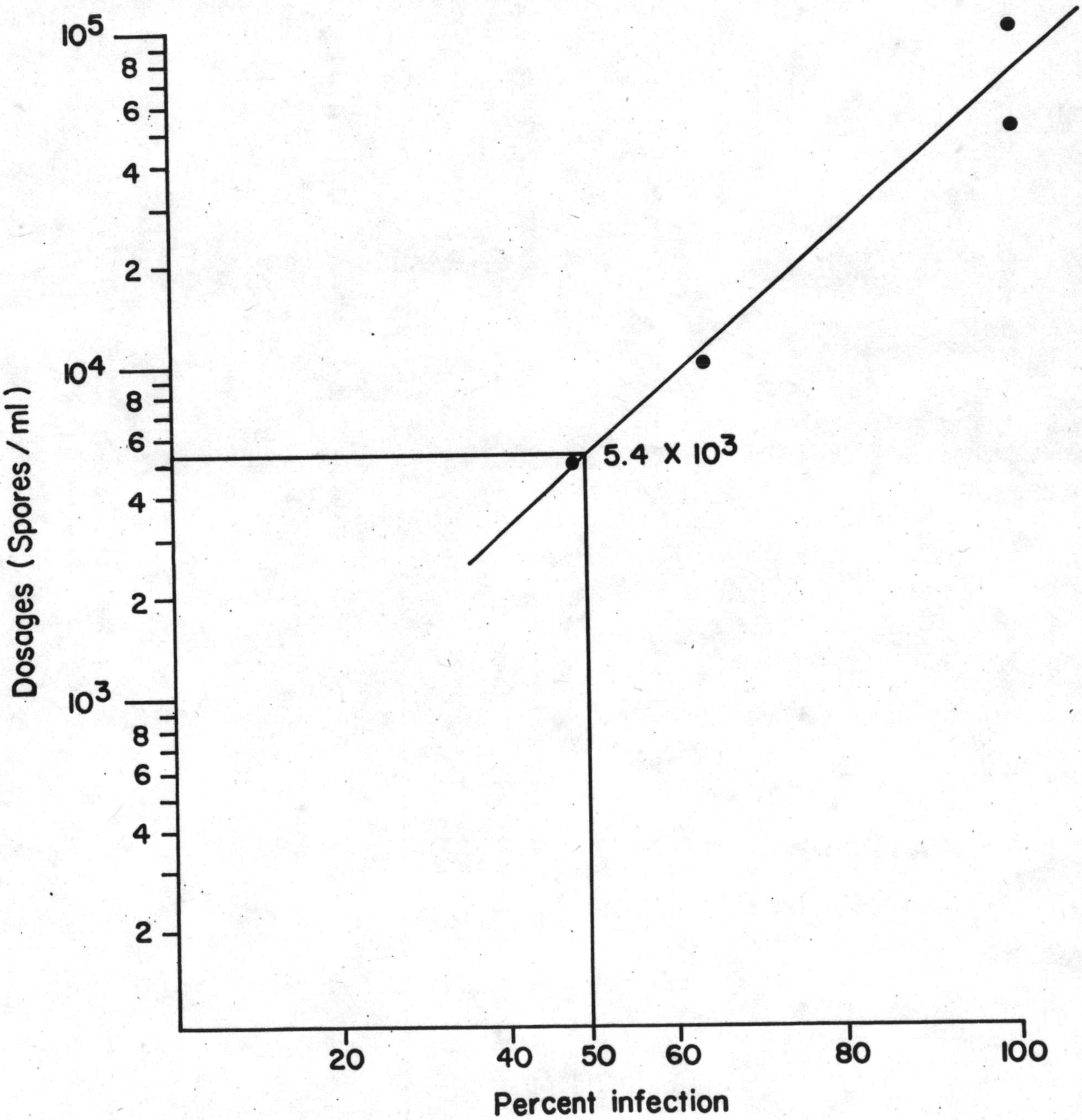


Fig 3f. Dose-response curve of class VII, 8 weeks after storage.

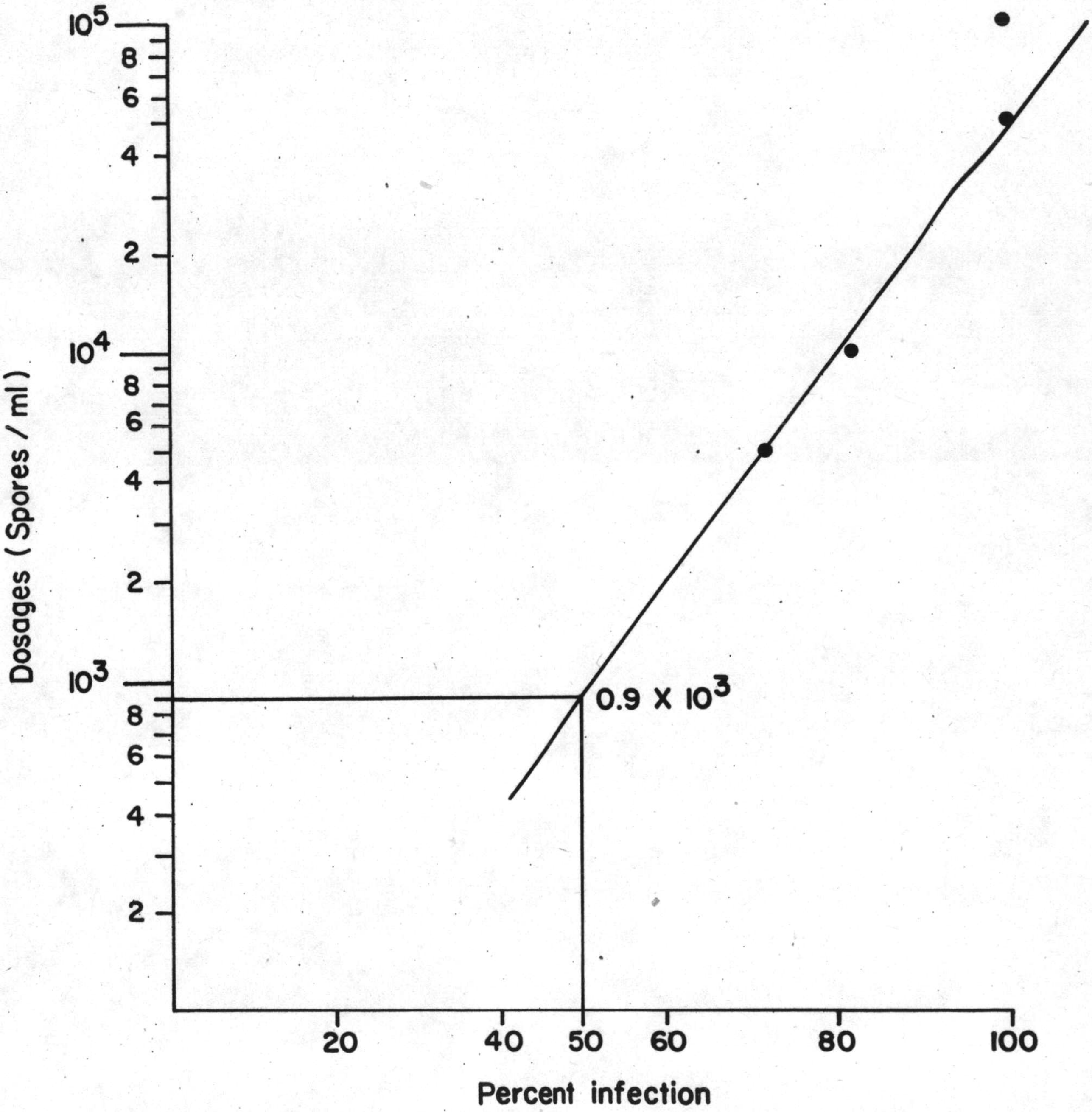


Fig 4a. Dose-response curve of class III, 12 weeks after storage.

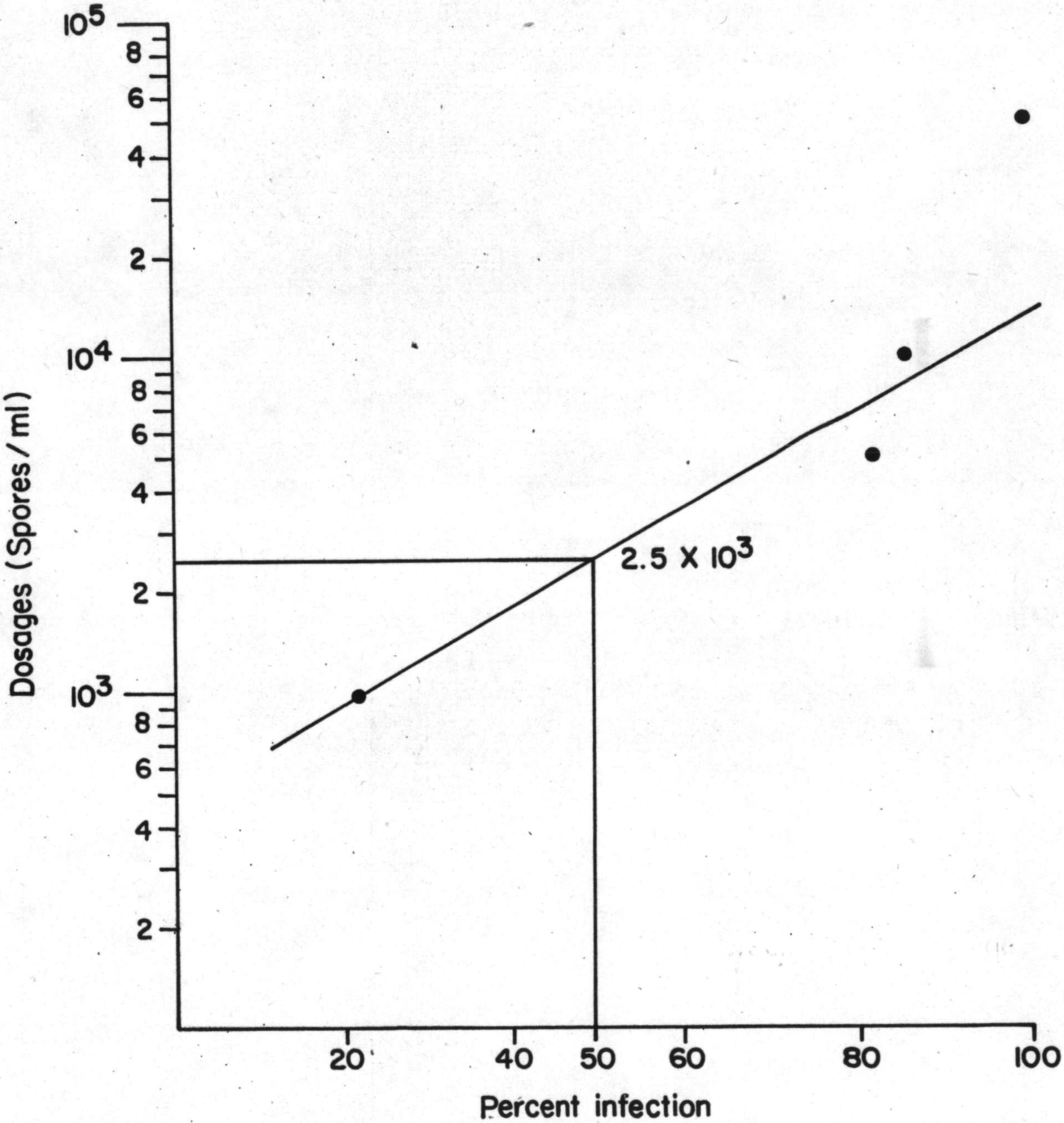


Fig 4b. Dose-response curve of class II-1, 12 weeks after storage.

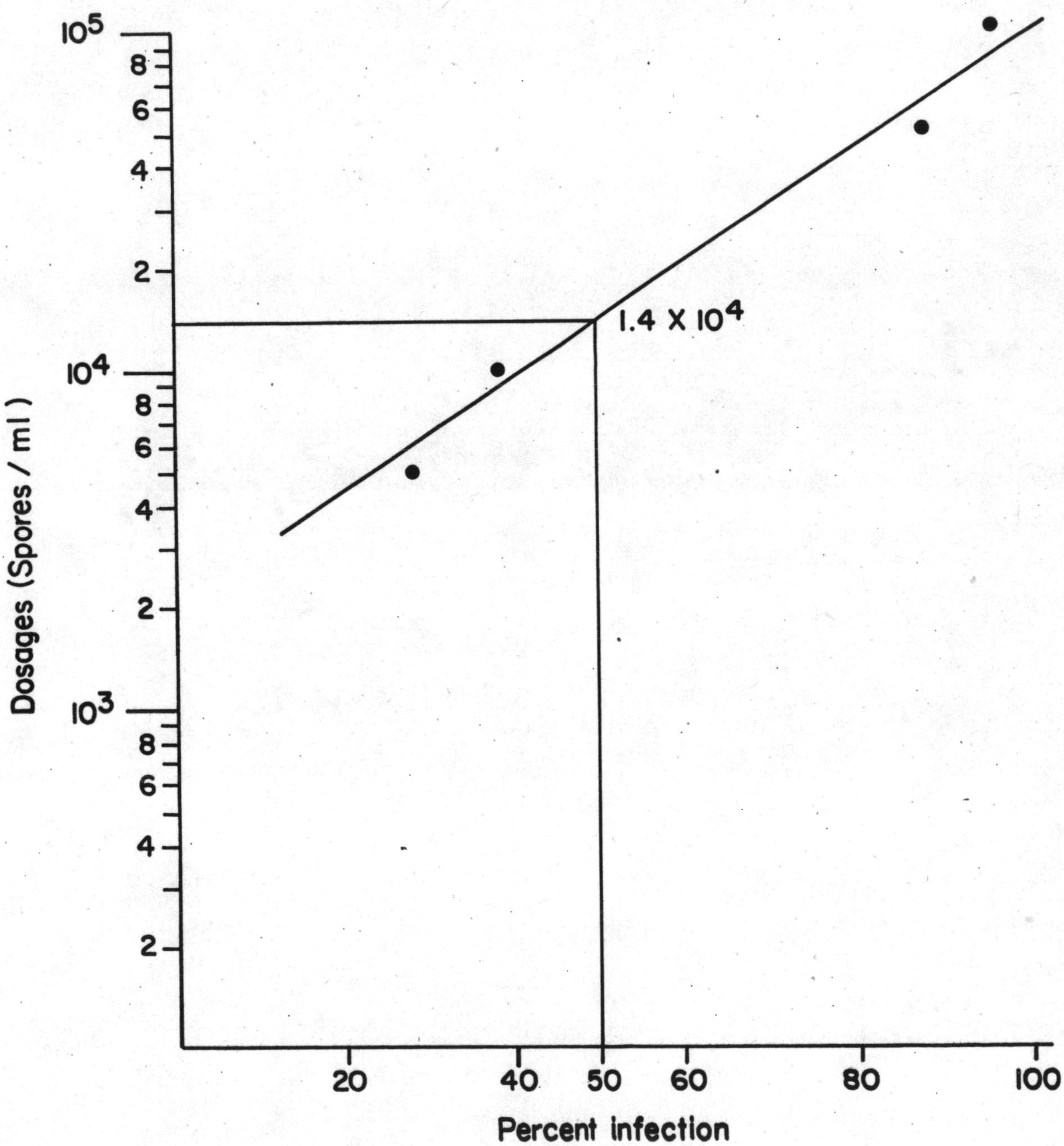


Fig 4c. Dose-response curve of class VI, 12 weeks after storage.

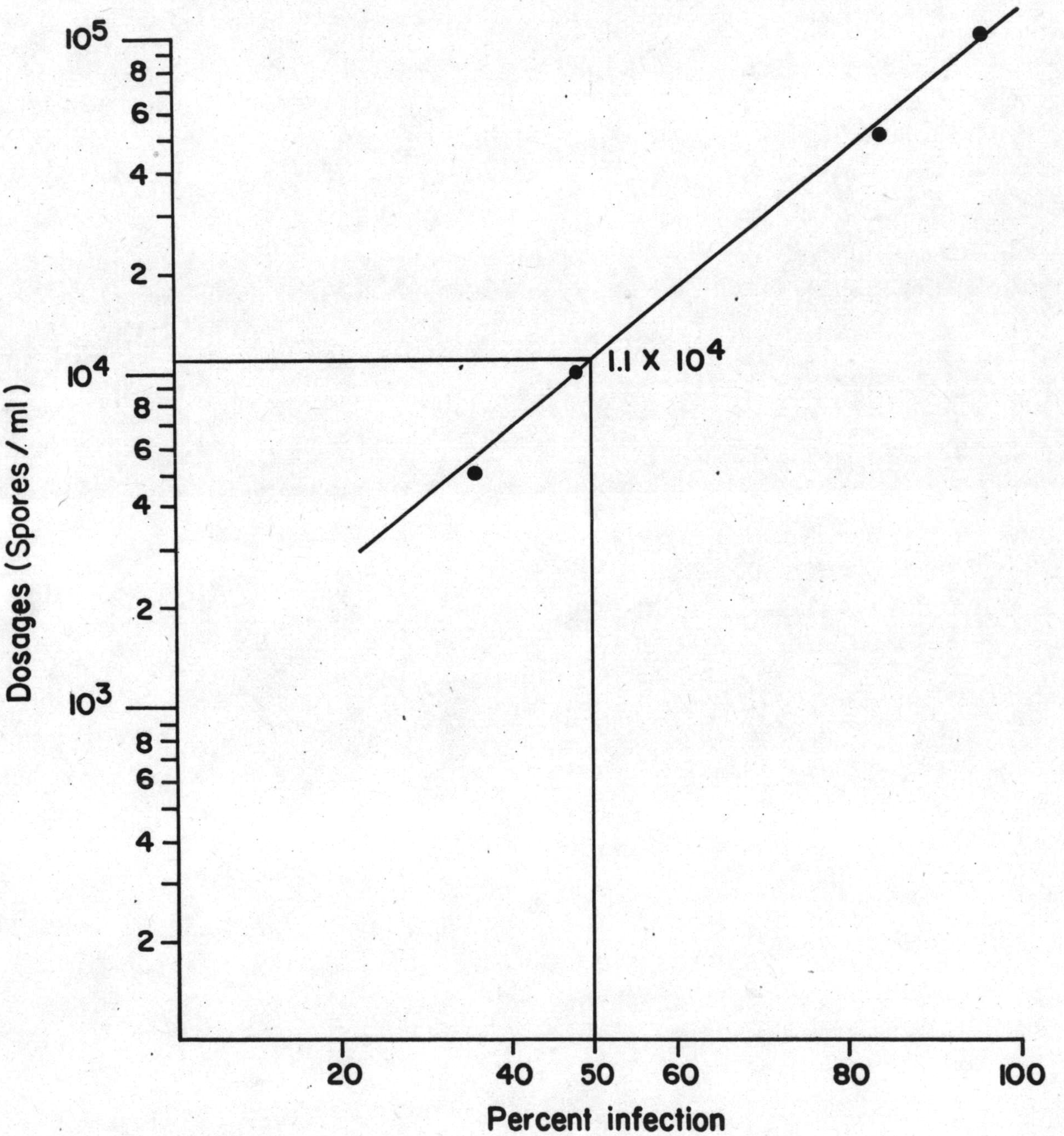


Fig 4d. Dose-response curve of class II-2, 12 weeks after storage.

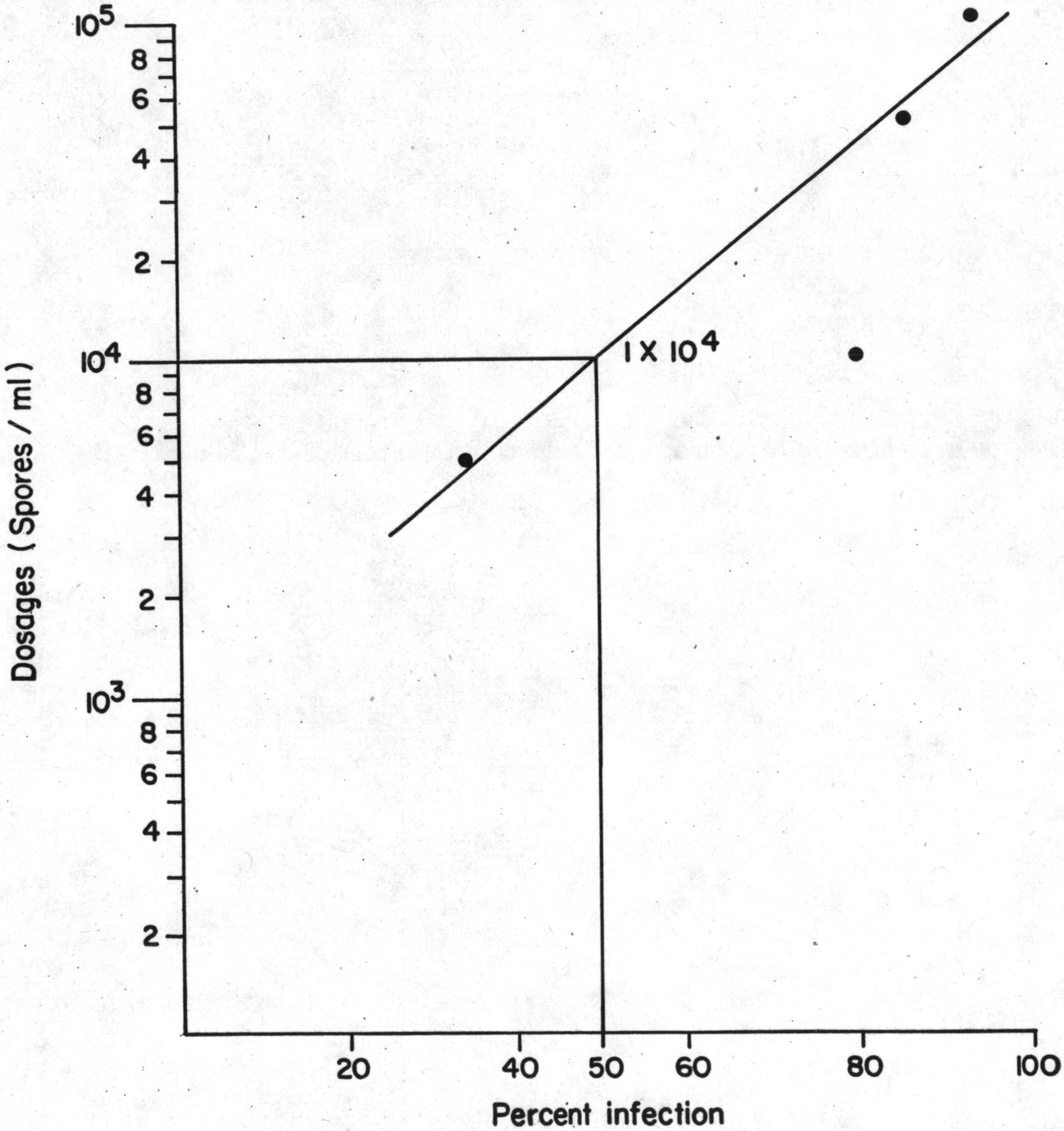


Fig 4e. Dose-response curve of class VII, 12 weeks after storage.

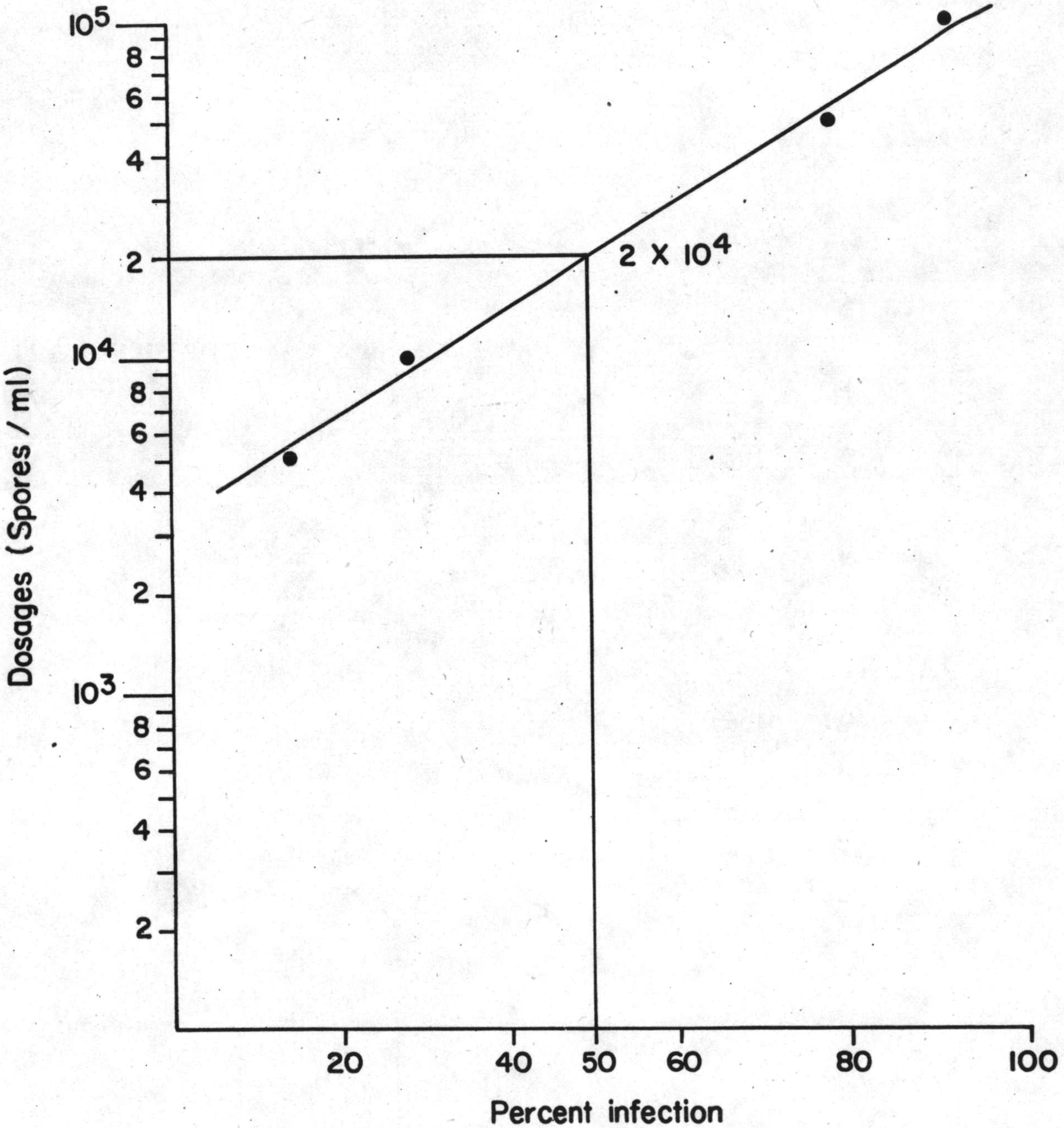


Fig 5a. Dose-response curve of class I, 16 weeks after storage.

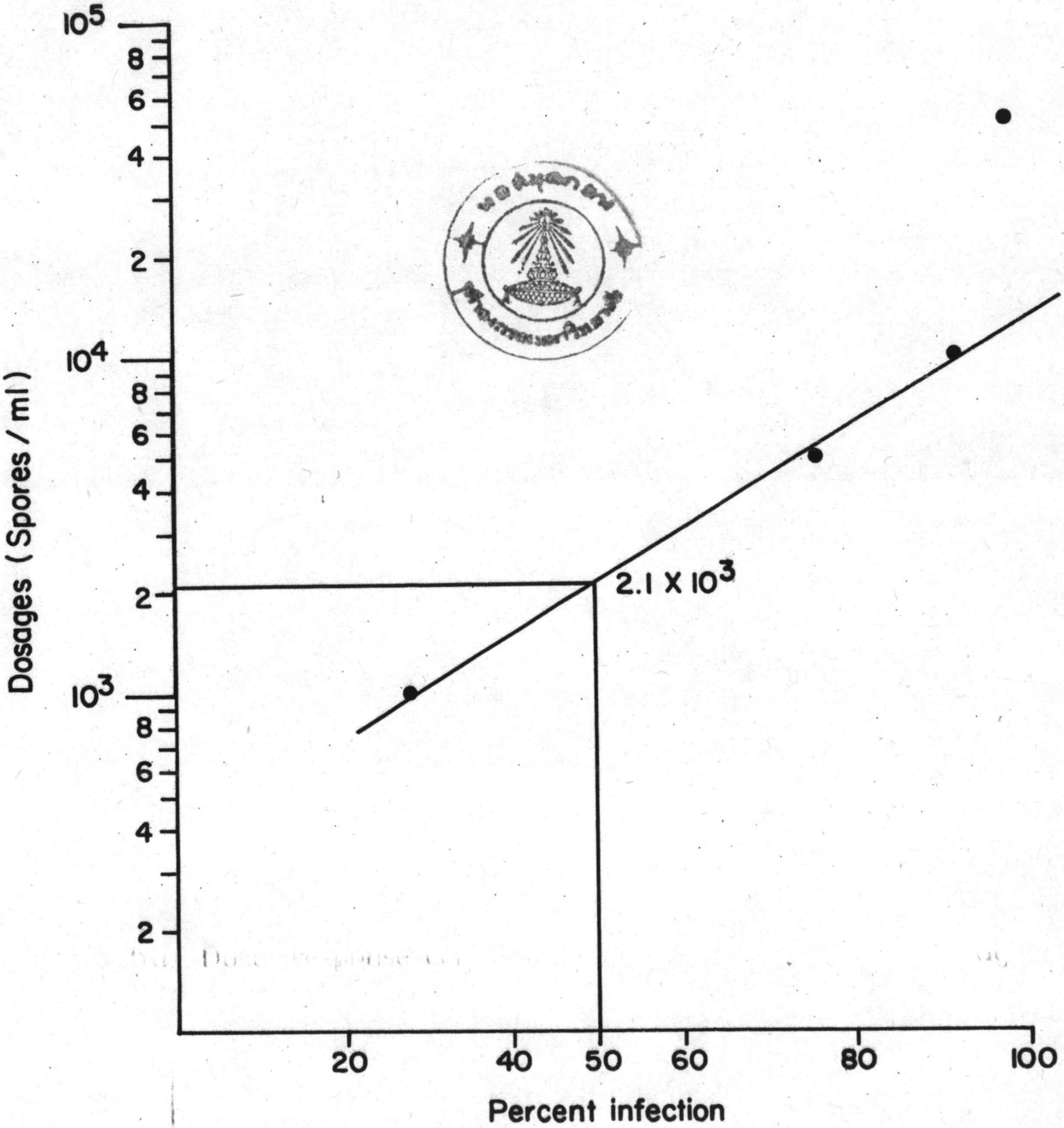


Fig 5b. Dose-response curve of class III, 16 weeks after storage.

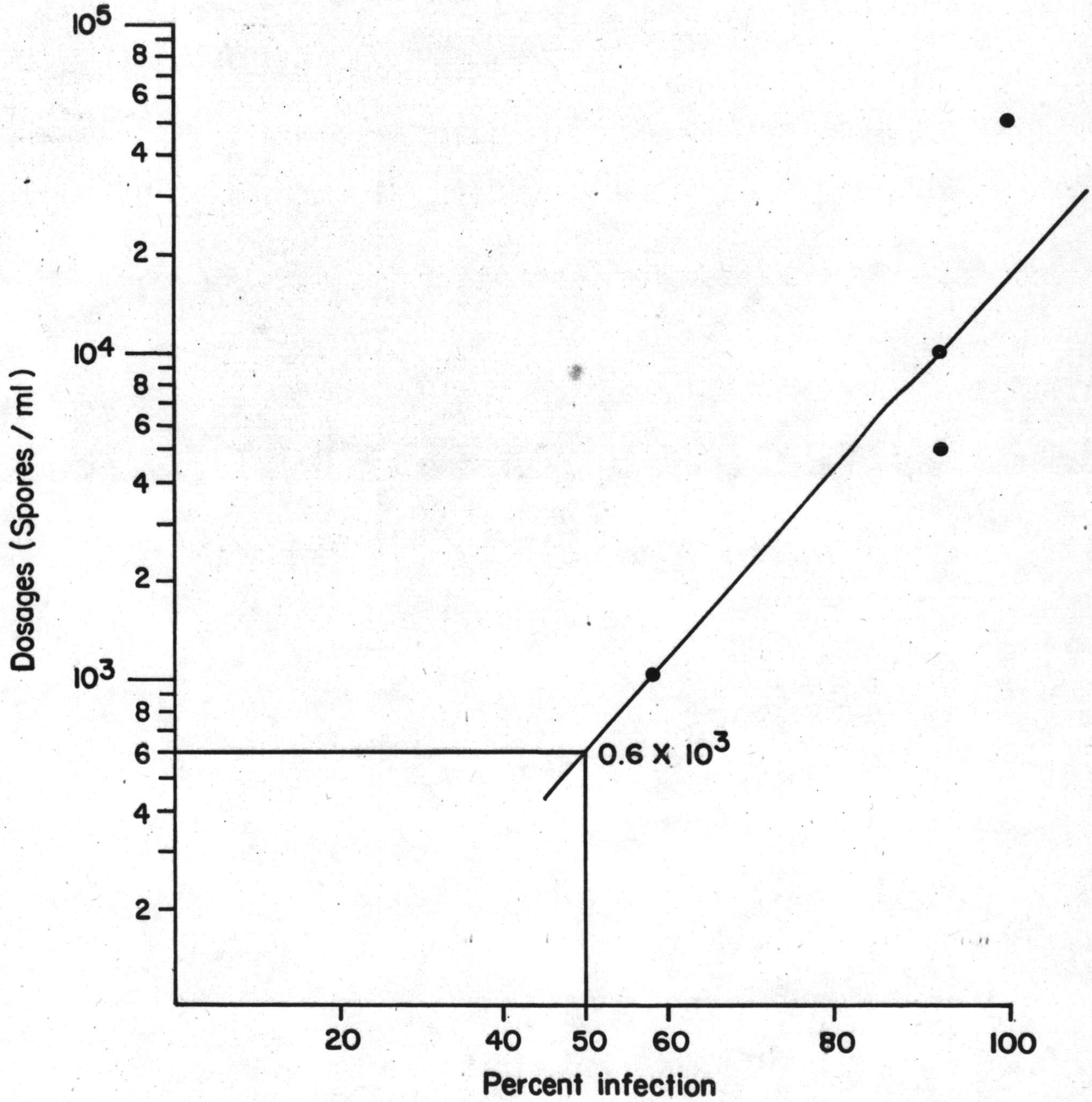


Fig 5c. Dose–response curve of class II-1, 16 weeks after storage.

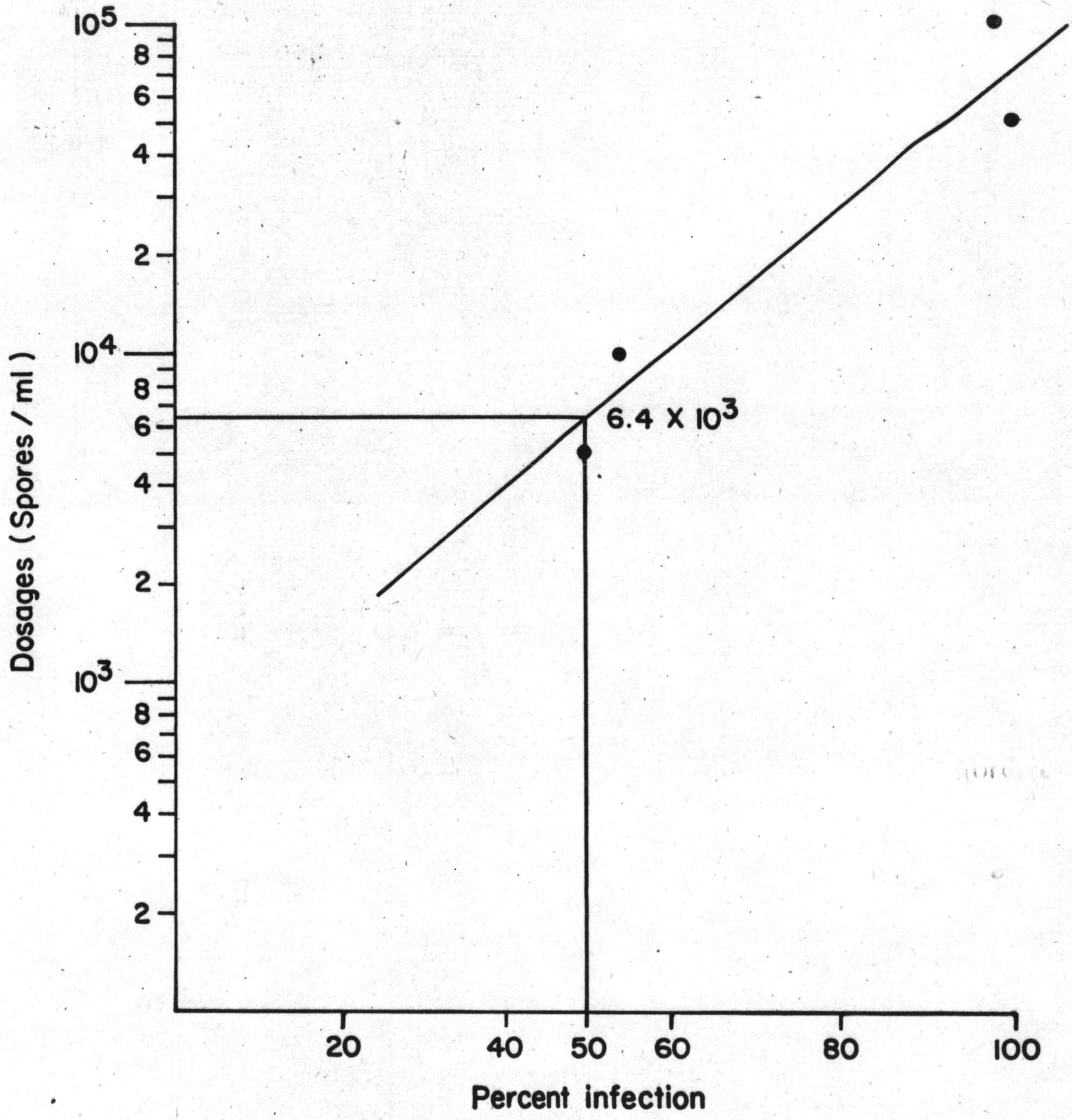


Fig. 5d. Dose-response curve of class VI, 16 weeks after storage.

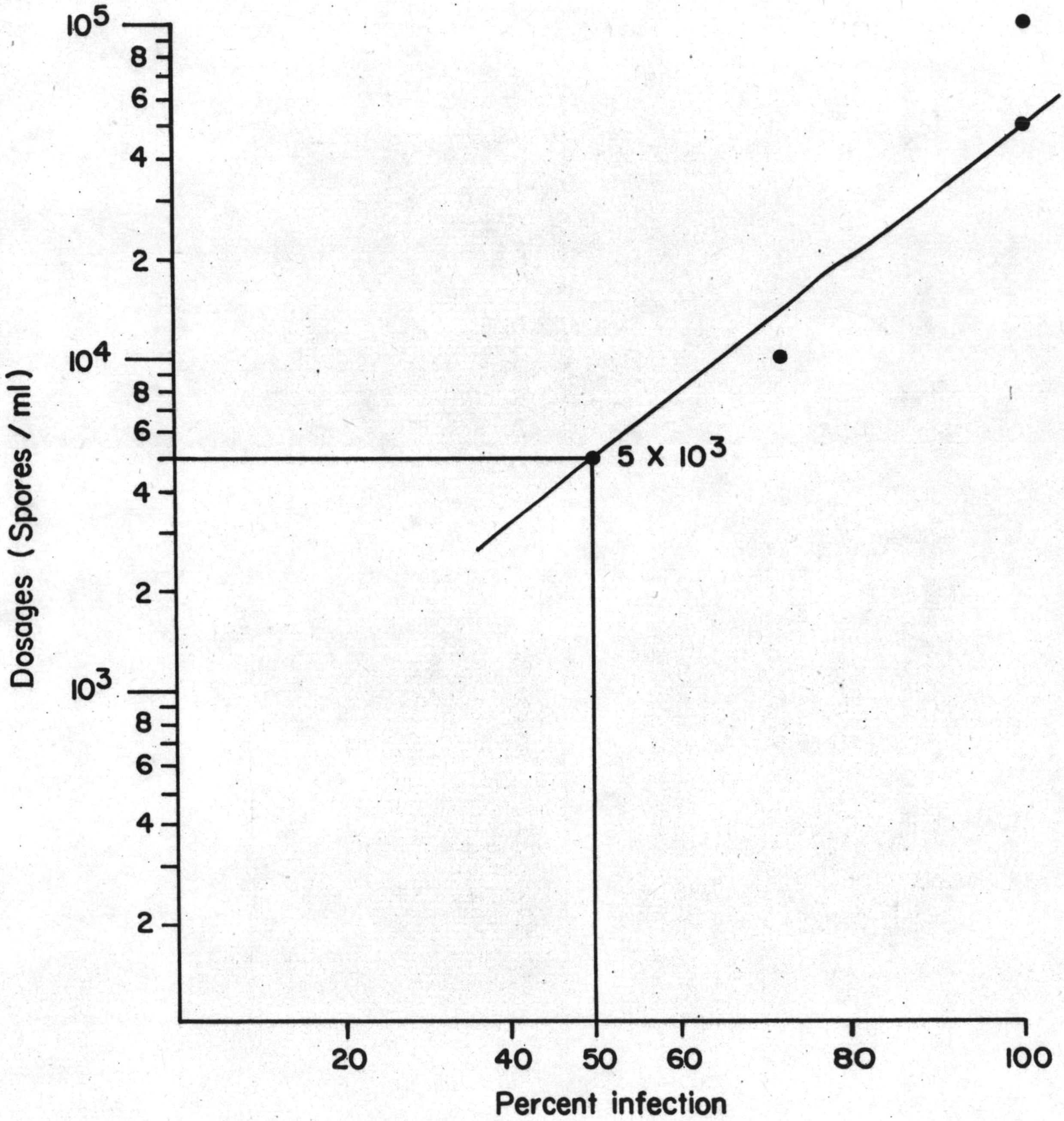


Fig 5e. Dose-response curve of class II-2, 16 weeks after storage.

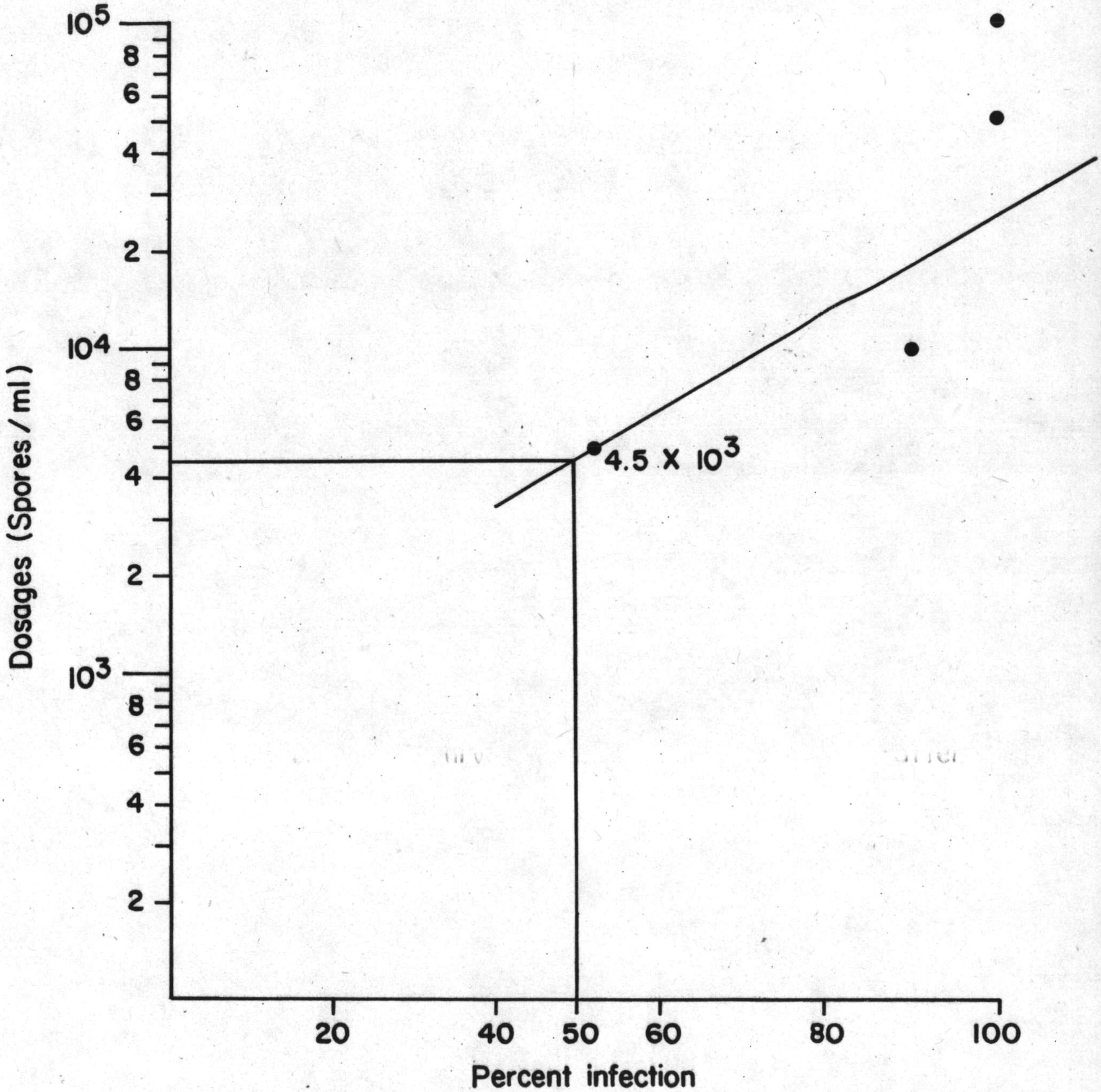


Fig 5f. Dose-response curve of class VII, 16 weeks after storage.

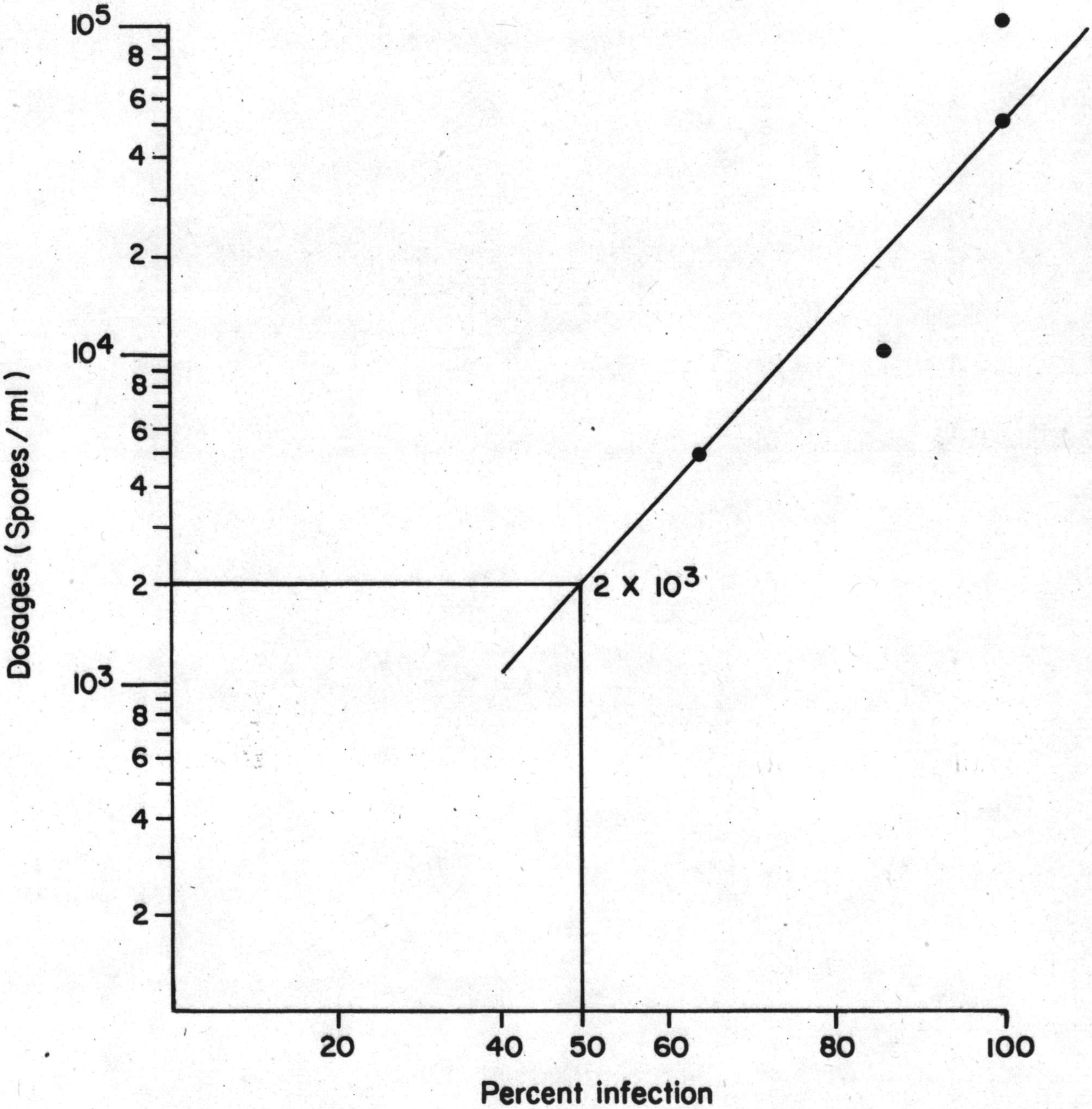


Fig 6. Relationship between IC_{50} and duration of storage (weeks).

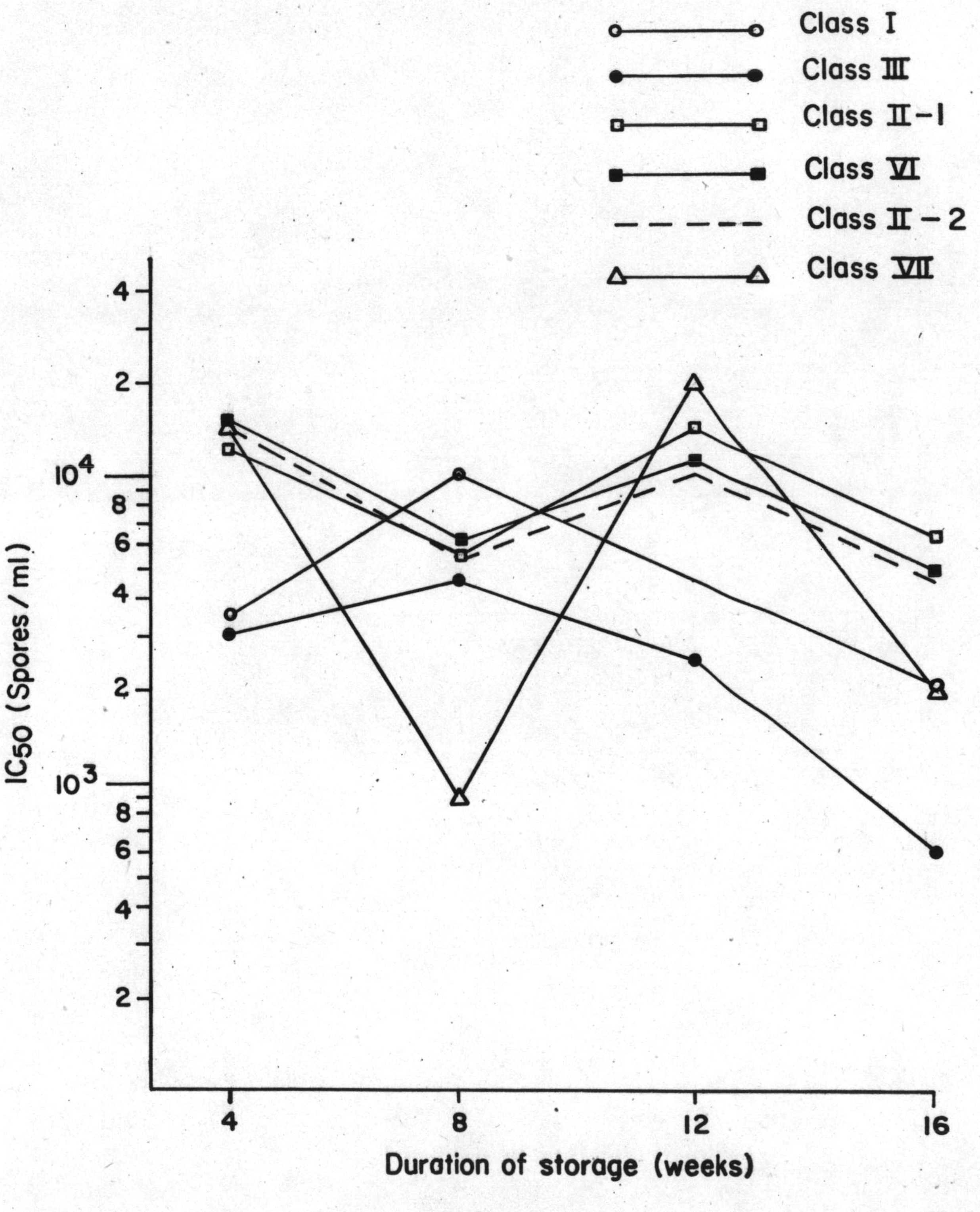
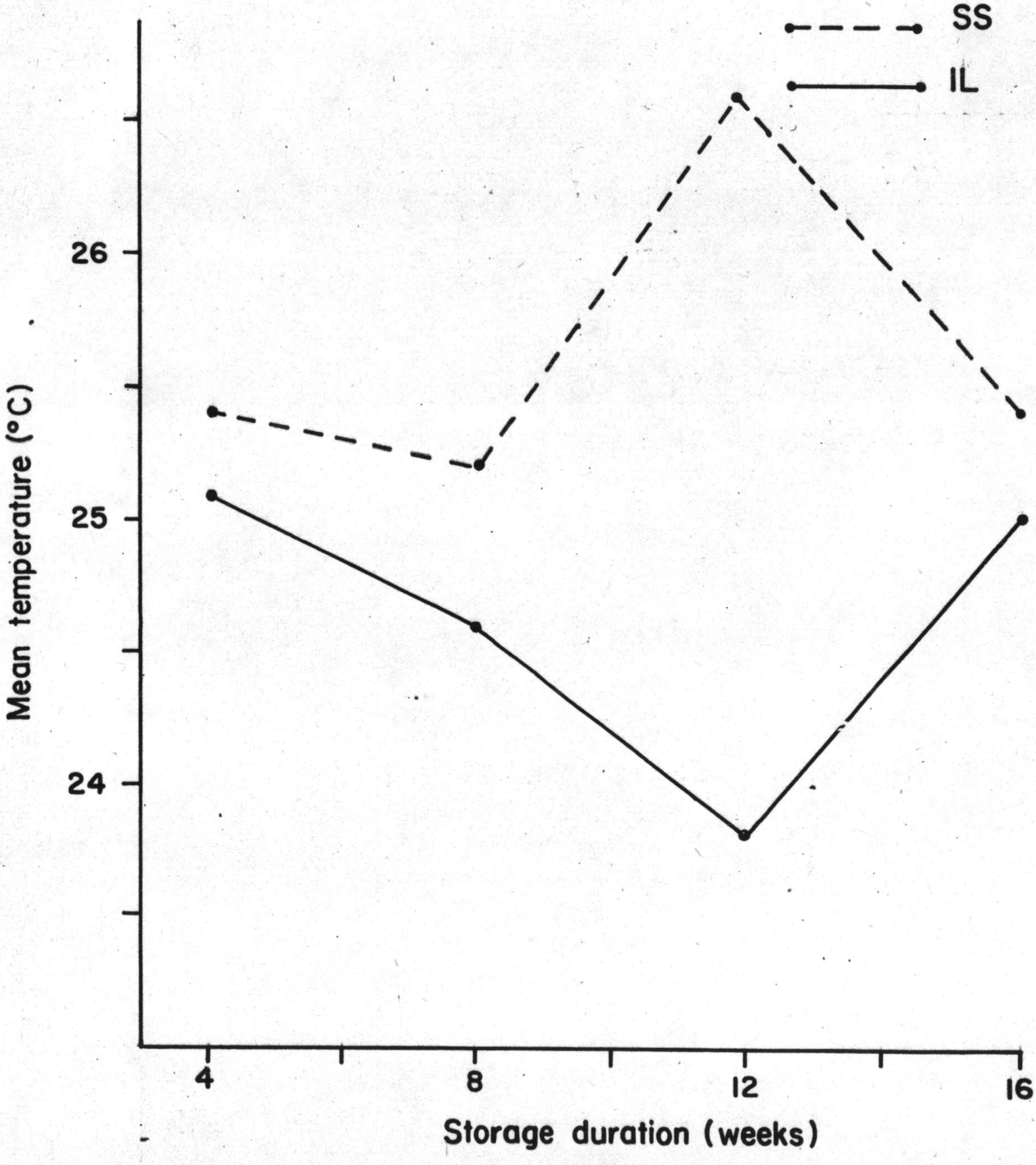


Fig. 7. Mean temperature (°C) of incubation days, the 2 days before infectivity tests of Helicosporidium spores, preserved in intact infected larvae (IL) and in the form of spore suspension (SS), after storages of various duration (weeks).



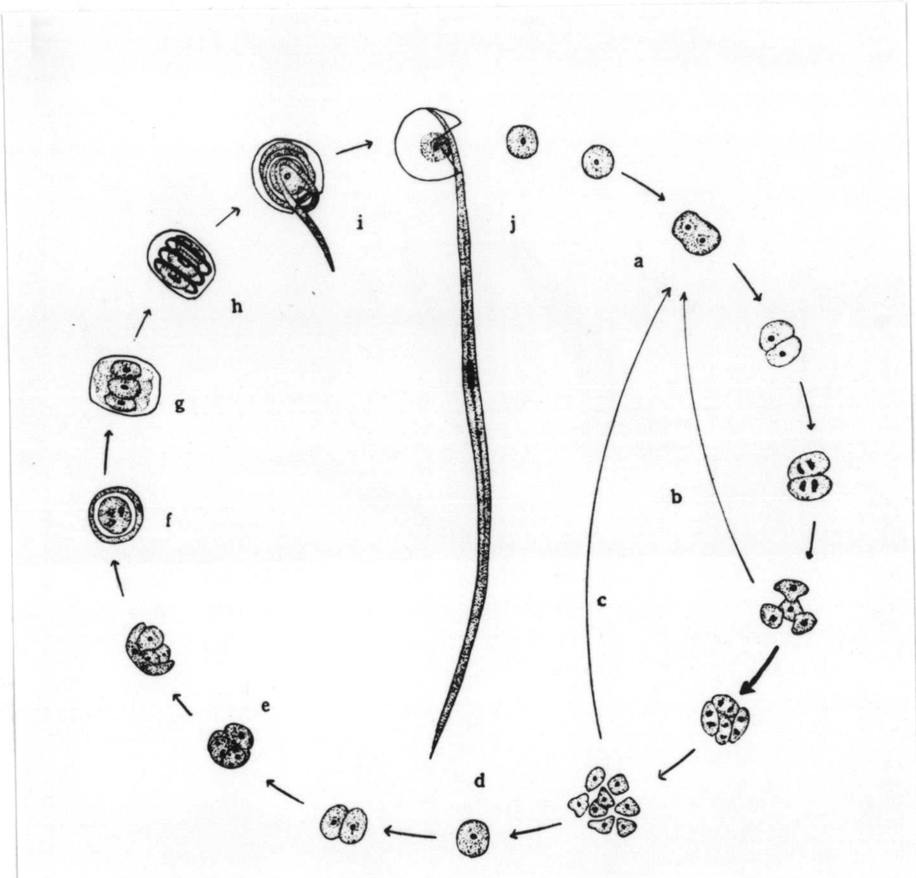


Figure 8. Diagram illustrating the probable development of Helicosporidium, x about 1600.

a-c, schizont and schizogony; d, sporont; e, three stages in formation of four-celled stage; f, hypothetical stage; g, young spore before the spiral filament is formed; h, mature spore; i, j, opening of spore and liberation of sporoplasms, a-h in living host larvae; i, j, in dead host body (from Kudo, 1971).

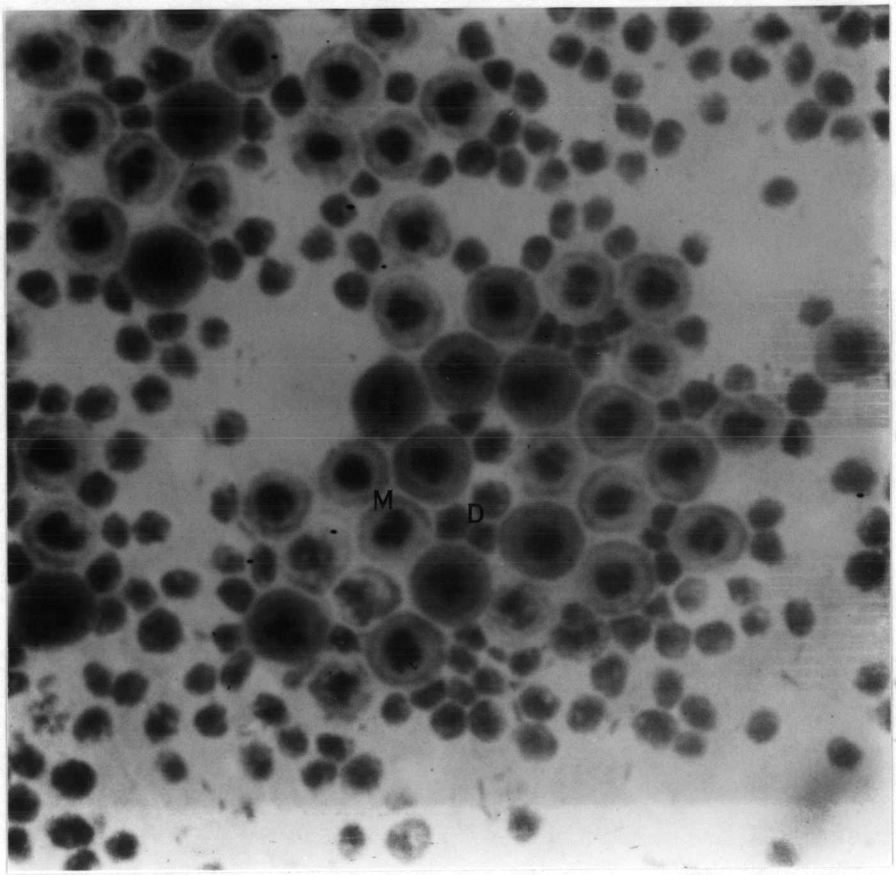


Figure 9. Giemsa stained smear of infected _Aedes aegypti_ larva, showing several developmental stages of _Helicosporidium_.

M = mature spores

D = other developmental stages

(1250 x oil immersion)

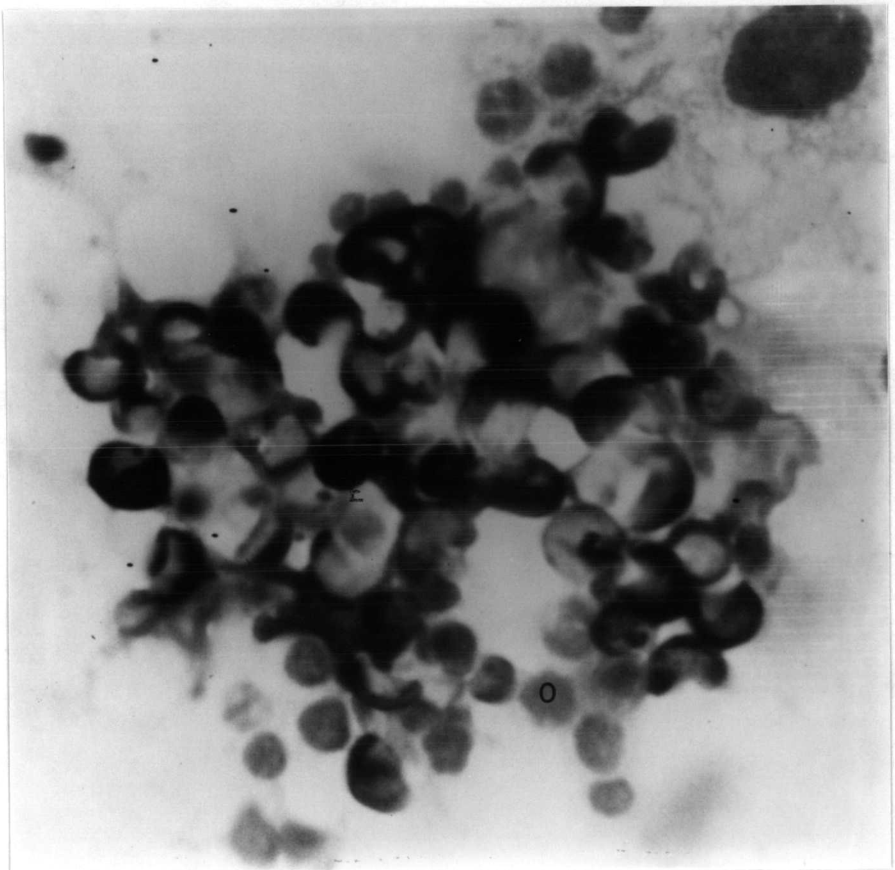


Figure 10. Giemsa stained smear of infected <u>Aedes aegypti</u> larva, showing melanin encapsulation (E) and sporonts (O) of <u>Helicosporidium</u>.

(1250 x oil immersion)

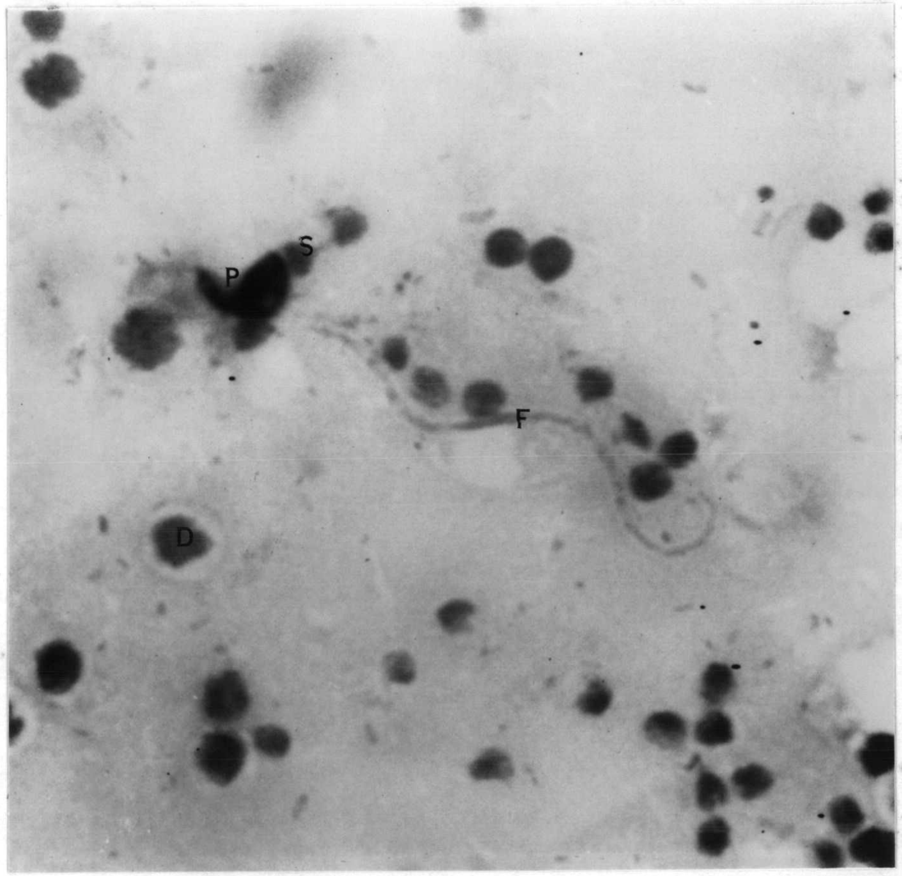


Figure 11. Giemsa stained smear of Helicosporidium infected Aedes aegypti larva, showing spore germination.

F = unrolled filament

P = ruptured melanized pellicle

S = sporoplasm

D = other developmental stages

(1250 x oil immersion)

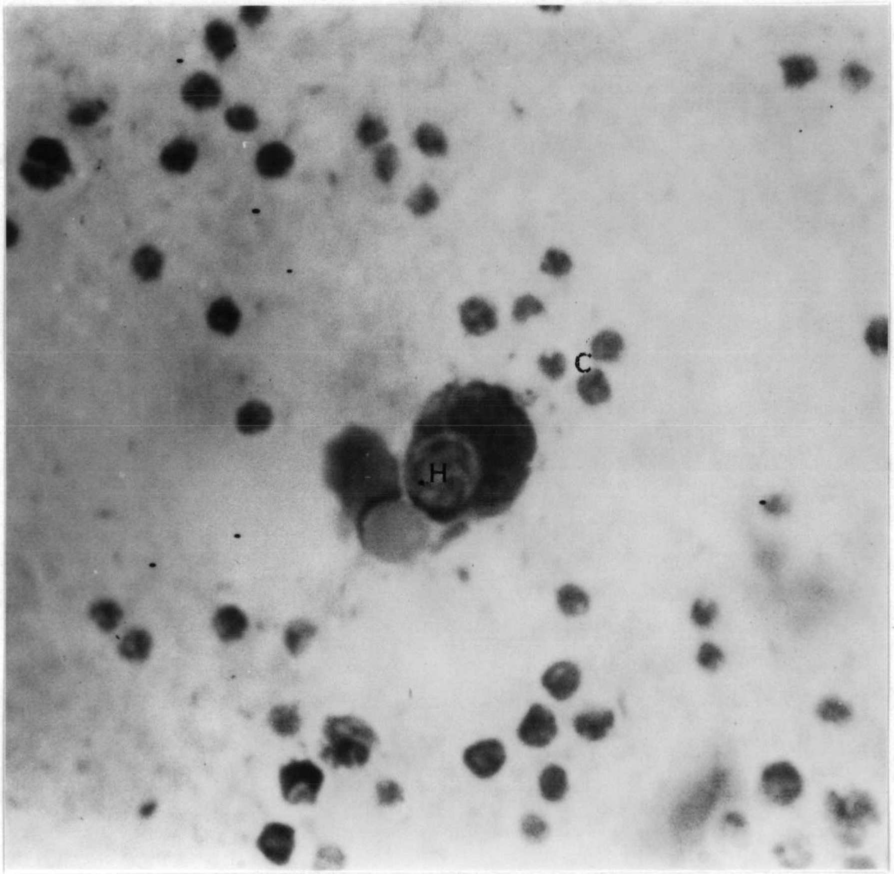


Figure 12. The developmental stages of Helicosporidium. Some were found in the host cell (H), and some were found in the hemolymph (C) of a giemsa stained smear of an infected Aedes aegypti larva.
(1250 x oil immersion)

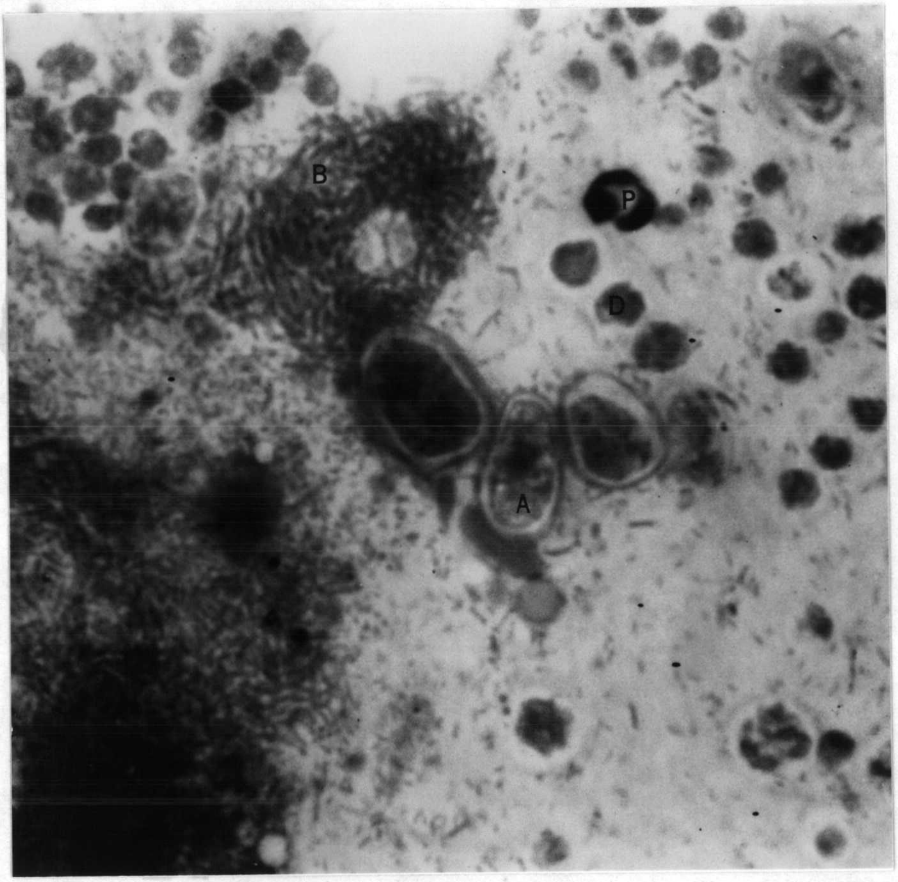


Figure 13. Giemsa stained smear of <u>Aedes aegypti</u> larva which was infected by several microorganisms including <u>Helicosporidium</u>, bacteria (B) and possibly commensal algae (A).

P = Ruptured melanized pellicle of <u>Helicosporidium</u>

D = Developmental stage of <u>Helicosporidium</u>

(1250 x oil immersion)

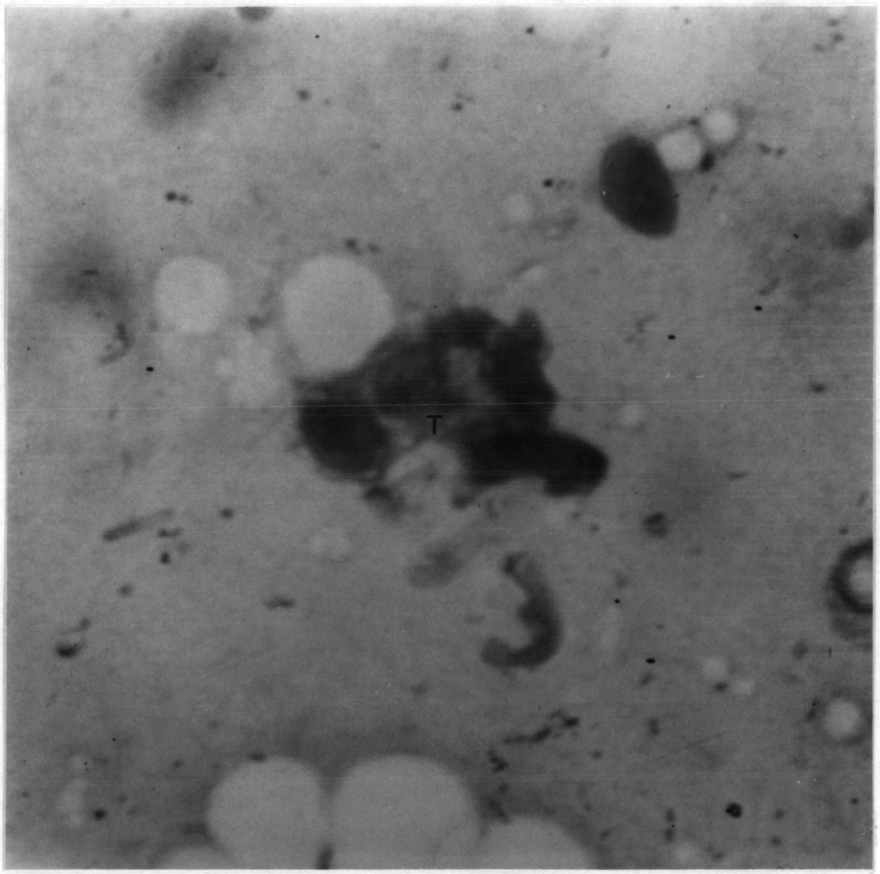


Figure 14. Giemsa stained smear of uninfected _Aedes aegypti_ larva as a control.

T = mosquito tissue

(1250 x oil immersion)