The Resistivity of Microorganisms to Thermal Inactivation by Dry Heat

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EXPERIMENTAL

I. Test Organisms

A. Test spores of Bacillus subtilis var. niger were used in the kinetic studies as a standard for comparison to the thermal resistivities of isolates obtained from soil.

It was found that the population of B. subtilis var. niger spores, normally a red-pigmented organism, also contained a number of mutants. These were:

1) Spores which formed less pigmented progeny.

2) Spores which were pigment-less, the resultant colonies being white.

3) Spores which formed minute colonies after a preliminary drying treatment at 45°C.

The above mutants were isolated and their thermal resistivities were compared to that of the original culture. Their incidence is not very high.

The method for obtaining spores, their methods for assay, and the support material for the determination of thermal resistivity were detailed in a previous report (Silverman and Dunn, 1965, Final Report Contract NSG-691, NASA).

A number of different assay media were also evaluated in this study and this phase is still being continued.

In general the method of evaluation includes placing a known number of heat-shocked spores on a membrane filter (S and S 2500) or a fiber-glass disc, drying at 45°C for 2½ hours, and placing the dried preparation over a silica-gel desiccant overnight at 20°C. After thermal exposure the membrane filters are placed on a nutrient agar - the standard being tryptone-glucose-yeast extract (TGE) agar. The fiber-glass filters are blenderized in distilled water and aliquots plated on TGE agar. Incubation was at 30°C, usually for one day but in some instances for as long as a week.

B. Isolates

The program of comparing the thermal resistivities of isolates obtained from various natural sources has been initiated and is being actively pursued. Microbial spores, vegetative species, and spores from actinomycetes obtained from a previous thermal-vacuum study are currently being evaluated. In addition, isolates from desert soils, air contaminants frommissile assembly plants, etc. are being prepared for examination. To date spores from five microbial species have been evaluated. The time required to perform the harvesting and purification of these spores has been lengthy. In the future the purification of these spores for screening purposes will be less thorough and only those species which are of high thermal resistivity will be prepared and studied in a more thorough manner.

II. Chambers

A. The first oven, which is designated as oven #1 has been described previously (Silverman and Dunn, Final Report 1965, NSG-691) and is essentially a precise convection chamber.

B. A newly designed chamber of improved design (oven #2) has been employed during this testing period. A schematic of it has been presented in Figure 1. The following criteria were established:

1) The temperature within the chamber be uniform to within $\pm$ 0.25°C. In actuality it is closer to $\pm$ 0.1°C.

2) The come-up time within the chamber is within 1°C of the final temperature within five minutes. This requirement will, of course, depend upon the desired temperature but has been met. (Figure 2)

3) The samples can be easily inserted and removed.

The apparatus consists of two hinged supports made from $1\frac{1}{2}$ inch maple. Heat is supplied to each half by a $8\frac{1}{2}$ inch by 11 inch 500 watt sheet heater which is constructed from a resistance nichrome wire (500 watt) sandwiched between two sheets of silicone rubber. Between the wood and the heater is a 1/8 inch asbestos pad and, on the other side of the heater, is a 1/8 inch copper plate. The inner chamber surface is therefore essentially

two copper plates. The copper plates are separated by a silicone rubber rim, each rubber rim being attached to a copper plate by Dow Corning 780 silicone rubber cement. The copper plate was attached to the wood block by tapered head screws, the surface of which was sealed with silicone cement. Temperature was measured by introducing four copper-constantin thermouples into the chamber through the bottom block. At times as many as ten thermocouples were used by introducing them through the rubber rim. Vacuum could be drawn, or gases admitted through three valves in the bottom block. The filters were introduced in the center of the chamber by a copper wire support which could be inserted or removed intact.

The temperature within the oven was controlled by a thermister probe placed between the heater and the asbestos pad in the lower block. The thermister probe activated a proportional temperature controller (Dynapac 15). The temperature was measured by a Minneapolis-Honeywell potentiometer Model 8686 and monitored by a Bausch and Lomb VOM-7 recorder ($\pm$ 0.1°C). The entire oven was enclosed in foil-covered styrofoam for additional insulation.

For vacuum experiments a 700mm Hg vacuum was drawn and air or gas admitted until the vacuum was raised to 100mm Hg. This procedure was repeated for a total of three times at which time vacuum pumping was stopped and gas admitted until atmospheric pressure was reached and then bled in at a very slow rate.

RESULTS

I. Spores and Assay Procedure.

The assay procedure using S and S 2500 membrane filters appeared to be satisfactory for approximately 10^5 spores or less. When a higher number of spores were used inaccurate results were noted due to the excessive bacterial load. Rim effects, that is a tendency for high survival around the outer edges of the membrane, were minimzed by the use of a rubber gasket in the suction apparatus, which constrained the spores so that they were never closer than 1/8 inch from the edge. The reason for the higher survival of the spores at the edge of the filter after heating is at present unknown. It is possible that it is an artifact due to localization of spores by vortexing with a resultant subsequent impingment of a higher number of spores at the outer edge which would appear to give a higher survival. This phenomenon was not experienced for lower numbers of spores.

As regards to the membrane, the vacuum required to impinge the spores onto the membrane during vacuum filtration did not seem to make any difference (from 200 to 700 Hg), nor did the surface of the filter. It was noted that a shiny and dull side was present on each membrane but no difference in recovery due to these surfaces was noted. Drying at 45°C overnight rather than for 2½ hours did not make any difference in survival.

Five assay media were evaluated for their efficiency in the recovery of Bacillus subtilis var. niger spores at 120°C. The results are presented in Table 1. TGE agar is the standard agar used in these experiments. None of the other agars tested appeared to be superior to TGE agar for this organism. Other agars will be evaluated in the future but it should be stated that any evaluation for a wide variety of micro-organisms is extremely time consuming. A limited number of different test organisms will be employed for evaluation but unless a significantly larger recovery is noted, TGE will be the media of choice.

II. Thermal Inactivation Curves of Bacillus subtilis var. niger.

The thermal inactivation curves of B. subtilis var. niger for oven #1 is presented in Figure 3. The data for 135°C may be inaccurate at short-time intervals due to the fact that in this oven the come-up time at 135°C is approximately 8-10 minutes. In Figure 4 the inactivation curve at 120°C and 135°C for oven #2 is presented and the data at 135°C is believed to be more precise than for oven #1. For comparison, data obtained from oven #1 at 120°C is also presented and it is seen to have lower values that of obtained in oven #2. The curves at 120°C is exponential after the first log cycle of destruction. The curve at 135°C, although seemingly linear on this scale, on a somewhat expanded scale is also non-exponential for the first log cycle of destruction. The reasons for this initial non-linearity may be due to a number of factors such as clumping, etc., or it may be an inherant property of the organism.

One interesting phenomenon was noted in Figure 4. In a study initiated to determine the effects of various inert gases on the rate of inactivation a sharp decrease in thermal resistivity at 120°C was noted. This procedure involved a vacuum-gas sparging step. It soon

became apparent that exposure of the spores to three successive periods of vacuum cycling of 30 seconds duration each was sufficient to subsequently sensitize B. subtilis var. niger spores to heat. For this reason further experiments to ascertain the effect of the presence of inert gases on thermal resistivity was discontinued.

The white and pink mutants of B. subtilis var. niger were found to be less resistant than those fully pigmented and the resistivity of those that form minute colonies will be evaluated in the near future.

III. Isolates

Spores from each of five aerobic Bacillus sp. were harvested and purified by washing and assayed at 120°C. These were originally obtained from soil experiments as described in methods. The data in Table 2 indicates that none were more resistant than B. subtilis var. niger.

DISCUSSION

From a practical standpoint oven #2 is clearly superior in design and performance to that of oven #1. The temperature uniformity is more uniform than oven #1 and the come-up time is appreciably shorter.

In the previous report two factors appeared to influence thermal resistance of spores of B. subtilis var. niger in oven #1. These were the effect of forced convection, which decreased survival, and the water content of the air. The dryer the air the lower the survival.

In preliminary experiments in determining come-up times both air and argon were comparable but greater than for helium (See Figure 2). Because of the lower heat capacity and the higher thermal conductivity the come-up time with helium was a quarter of that of air or argon.

Although the temperature profile in oven #2 was considered isothermal this was in a plane adjacent to the filters. Even though the vertical distance within the chamber was small it took a finite time for the vertical temperature pattern to approach uniformity (Table 3). It was assumed in these studies with the second chamber that convection was minimized and that energy transfer was essentially by either radiation or by diffusion.

The effect of drawing a vacuum and the reintroduction of air or helium to the chamber, which resulted in decreasing survival, introduced a number of other variables into the experiments. This observation, plus the fact that forced convection was noted to have decreased survival in oven #1 indicated that in some manner, during the initial subjection of the spores to elevated temperatures a critical event(s) occurs. This alteration of spore integrity is reflected in a lower thermal resistivity. Two speculations appear to be of most promise. This effect was not noted in experiments with forced convection at room temperature or by the vacuum treatment at room temperature but only at high temperatures. It is conceivable that at an elevated temperature water or some other volatile component is removed from the spore which further sensitizes the spore to heat. The water which is not removed by preliminary desiccation may be necessary for the maintenance of the integrity of the constituents of the spore, and which may be responsible for maximal thermal resistivity. This effect will be pursued in future research. It is interesting though that a related effect had been noted by Davis, Silverman, and Keller (Applied Microbial 11, 202, 1963) for spores heated in vacuum above 60°C, for vegetative cells (Silverman, unpublished) at temperatures above 45°C, and was postulated by Bruch (Life Sciences and Space Research, North-Holland Publ. Co., Amsterdam; A. Dollfus, ed., p. 357). The relatively short vacuum-air exposure treatment, lasting only a total of a minute and a half, appeared to be capable of permanantly sensitizing the entire spore population since the

slope of the inactivation curve did not change. The second postulate is that the vacuum-air treatment may cause a premature germination state in the spores which increases their susceptibility to heat. The absence of water in this system would prevent a further and more obvious germination from occuring. This second postulate will also receive some attention in future investigations.

There are a number of approaches to the evaluation of data obtained from thermal inactivation studies. Assuming a logarithmic inactivation pattern one may state mathematically that:

$$\log \frac{N}{N_o} = -\frac{K't}{2.303} = -Kt = -\frac{t}{D}$$

Where N = number of surviving spores at time t

N_o = initial number of spores at time t_o

K' = rate constant; $K = \frac{K'}{2.303}$ = slope of survival curve

t = time

D = 1/K = time to destroy 90% of the spores.

This linear relationship has been accepted in practice over a small temperature range by most investigators but has recently been questioned by others--at least for a number of organisms. In an attempt to expand studies to wider temperature ranges more recent attempts have been devoted toward applying chemical reaction kinetics to survival data. The Arrhenius equation is just such an attempt and which is:

$$K' = Ae^{-Ea/RT}$$

where Ea is termed the activation energy, R being the universal gas constant, A the frequency factor, T the absolute temperature, and R the universal gas constant. Normally, by plotting $D/2.3 = K'$ versus the reciprocal of the absolute temperature, a straight line having a slope Ea (2.3R) is obtained.

Another method of analysis is that of the Absolute Reaction Theory of chemical Kinetics so that:

$$K' = K''' \cdot \frac{K'' T}{h} e^{\Delta S^{\neq}/R} e^{\Delta H^{\neq}/RT}$$

where K''' is the probability that an activated complex will not return to the state of reactant molecules rather than to proceed to that of the reaction products; K'' is Boltzmann's constant, T is the absolute temperature, ΔS is the entropy of activation; ΔH is the enthalpy of activation, and h is Planck's constant.

The theory implies that before forming a product the reactants must attain an activated state - which determines the rate of reaction. For spores, a spore would reach an activated state before it is destroyed. Free energy ΔF is related to ΔH and ΔS by the relationship:

$$\Delta F^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq}$$

It is assumed that by obtaining values for these various terms a greater insight into mechanisms underlying thermal inactivation can be obtained and that known chemical reactions can serve as the model for these mechanisms.

In this study values for these various terms have been derived. In actuality their application is of limited usefulness as regards to explaining precise mechanisms but, it is hoped that as more data is obtained, and the variables become elaborated that additional insight will be derived. Some representative values are reported for this study in Table 4 and the Arrhenius plot is given in Figure 5. The data obtained in this study is compared to that of Koesterer (Nasr-31, Final Report, 1962; Developments in Industrial Microbial. 6, 268, 1964). The D values are presented in Fig. 6.

The calculations used in Table 4 is derived from both ovens 1 and 2. This is not considered to be a significant source of error since, at 120°C, although the shoulders for the inactivation curves differ, the slopes are essentially equal. In radiation a number of equations have been derived for non-exponential curves of this nature. These equations will be further evaluated for their application to dry heat in the next report.

Analysis of the kinetic data for dry heat indicates that the entholpies are small, characteristic of simple chemical reactions, and that the entropies of activation have small positive or negative values. This data supports the hypothesis that molecular aggregates, necessary for the maintenance of viability of the cell, are not inactivated by dry heat by a large number of randomizing events such as the uncoiling of protein micelles, etc.. Typical kinetic data for wet heat normally reflects large configurational changes (Pollard and Reume, Biophysics 32, 278, 1951). Pollard and Reume noted, for the T phages, a much lower ΔS and ΔH for dry heat than for wet heat, and caution that this type of analysis does not lend itself to a precise analysis of structural changes. Pflug (Food Technol. 14, 483, 1960; see also Phiel et al; Microbiol. Abs. A19, p. 5 1963) found that superheated steam was less effective for spores of Bacillus subtilis strain 5230 than saturated steam. The Z value in their study was found to be 42°F, in this study it was 32°F. Koesterer (op. cit.) obtained different values than those noted in this study. This may very well be due to strain differences. One strain of B. subtilis var. niger in the authors laboratory has yielded values similar to his.

FUTURE EXPERIMENTS

1. The thermal resistivities of a wide spectrum of organisms will be determined by screening procedures. This will include those organisms found in soil, or air contaminants. The most resistant organisms will be studied in more detail and kinetic data will be obtained. Bacillus subtilis var. niger will be used as a standard.

2. The effect of an initial vacuum exposure to spores will be investigated. Certain aspects of germination and outgrowth will be examined.

3. The effect of equilibrating spores of B. subtilis var. niger at different water activities will be ascertained. If this factor proves to be important then a variety of isolates will also be studied.

4. Recovery media will continue to be evaluated.

5. The effect of the composition of the sporulating media on thermal resistivity to dry heat will be determined.

Table 1. Recovery of *Bacillus subtilis* var. *niger* at 120°C.

Media	Exposure (Hours)	Survival Fraction
Brain Heart infusion agar	2	1.4×10^{-2}
Nutrient agar	1	1.7×10^{-1}
Plate Count Agar	2	1.7×10^{-1}
Tryptone-glucose-extract	1	3.3×10^{-1}
	2	1.8×10^{-2}
	3	3.2×10^{-4}
Trypticase-Soy +0.5% yeast extract	2	1.8×10^{-2}

Table 2. Thermal Resistivity at 120°C of Five Isolates From Soil.

Isolate	Period of Exposure (Hours)	Survival Fraction
39A	2	5.6×10^{-3}
50A	2	1.4×10^{-4}
606A	2	$< 10^{-5}$
607A	2	$< 10^{-5}$
984	1	7.2×10^{-3}
	2	7×10^{-4}
Bacillus subtilis	1	3.3×10^{-1}
var. *niger*	2	1.8×10^{-2}

(From Table 1.)

Table 3. The Horizontal and Vertical Temperature Distributions* in Oven #2 at 120°C.

1. Horizontal distribution in center plane

Distance along / Distance across	1.5in	4.0in	6.5in	9.0in
1.5in	-0.1°C	+0.05°C	+0.05°C	-0.15°C
4.0in	-0.2	+0.1	+0.05	-0.1
6.5in	-0.2	+0.05	+0.05	-0.2

2. Vertical distribution

Distance above center plane	0.125in	+0.3°C
Center plane	0.0in	0.0°C
Distance below center plane	0.125in	-0.2°C

*The values indicate the average deviation from the mean oven temperature.

Table 4. Kinetic Values Calculated from the Data in this Study And from the Data of Koesterer (1964)

	T (°C)	D (hr)	K' (sec^{-1})	Ea/R (°C)	Ea (cal/mole)	A (sec^{-1})	Z (°F)
This study	106	4.4	1.4×10^{-4}	17,700	35,400	10^{16}	32
	120	0.8	8.0×10^{-4}			10^{16}	
	135	0.13	5.1×10^{-3}				
Koesterer	115	2.5	2.55×10^{-4}	12,900	25,800	10^{11}	49

	T (°C)	$\Delta F^{\neq}$ (cal/mole)	$\Delta H^{\neq}$ (cal/mole)	$\Delta S^{\neq}$ (cal/mole °K)
This study	106	29,000	34,600	14.8
	120	28,000	34,600	14.5
Koesterer	115	29,300	25,000	-11.1

Figure 1. Schematic of Oven #2

Key

A. Copper wires for supporting filters

B. Coarse wire screen support

C. Fiberglass insulation

D. Silicon rubber rim

E. Copper plate

F. Sheet heater

G. Asbestos pad

H. Wood insulation

I. Valve outlets

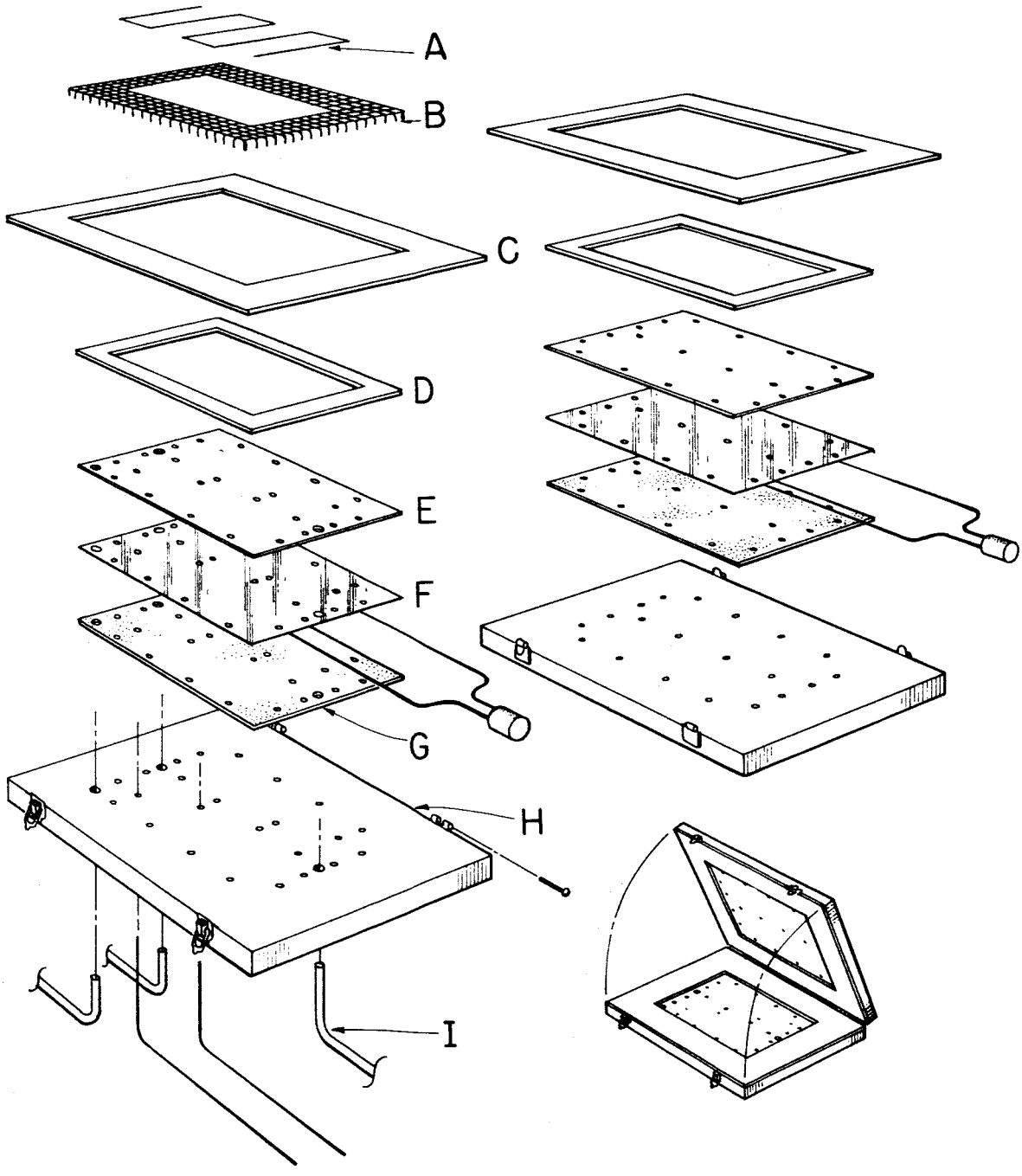

A
B
C
D
E
F
G
H
I

Figure 2. Come Up Characteristics
Of Oven #2 at 120°C

Key

Normal condition air

Vacuum-forced convection treatment with air and argon

Vacuum-forced convection treatment with helium

Normal condition, oven #1

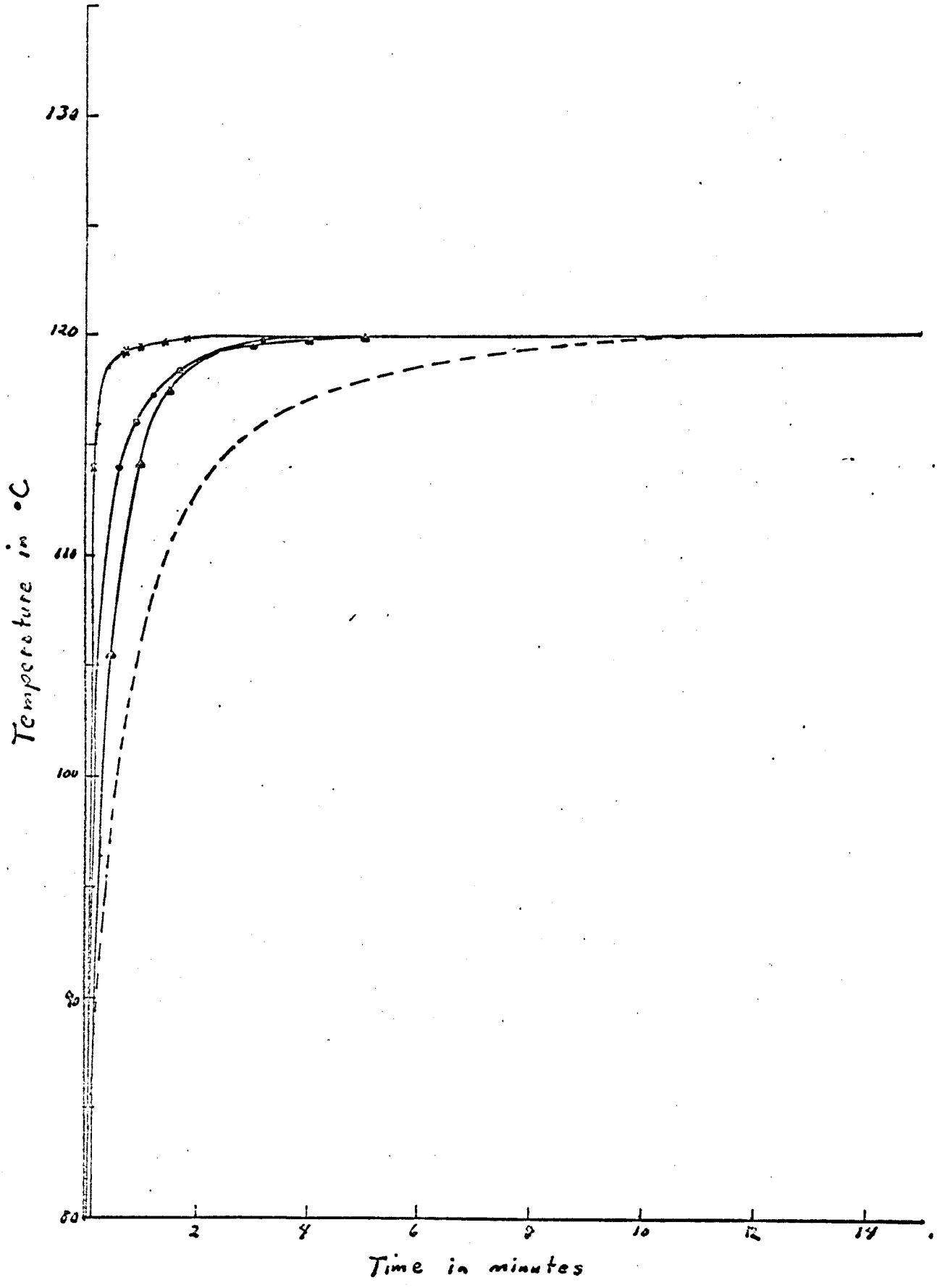
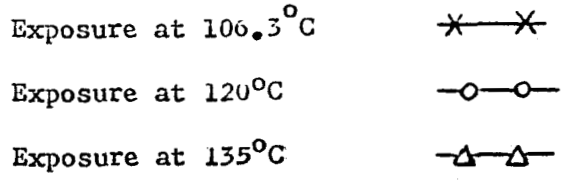

130
120
110
100
90
80
Temperature in °C
2
4
6
8
10
12
14
Time in minutes

Figure 3. Survival Curves for Air
For Bacillus subtilis var. niger in Oven #1



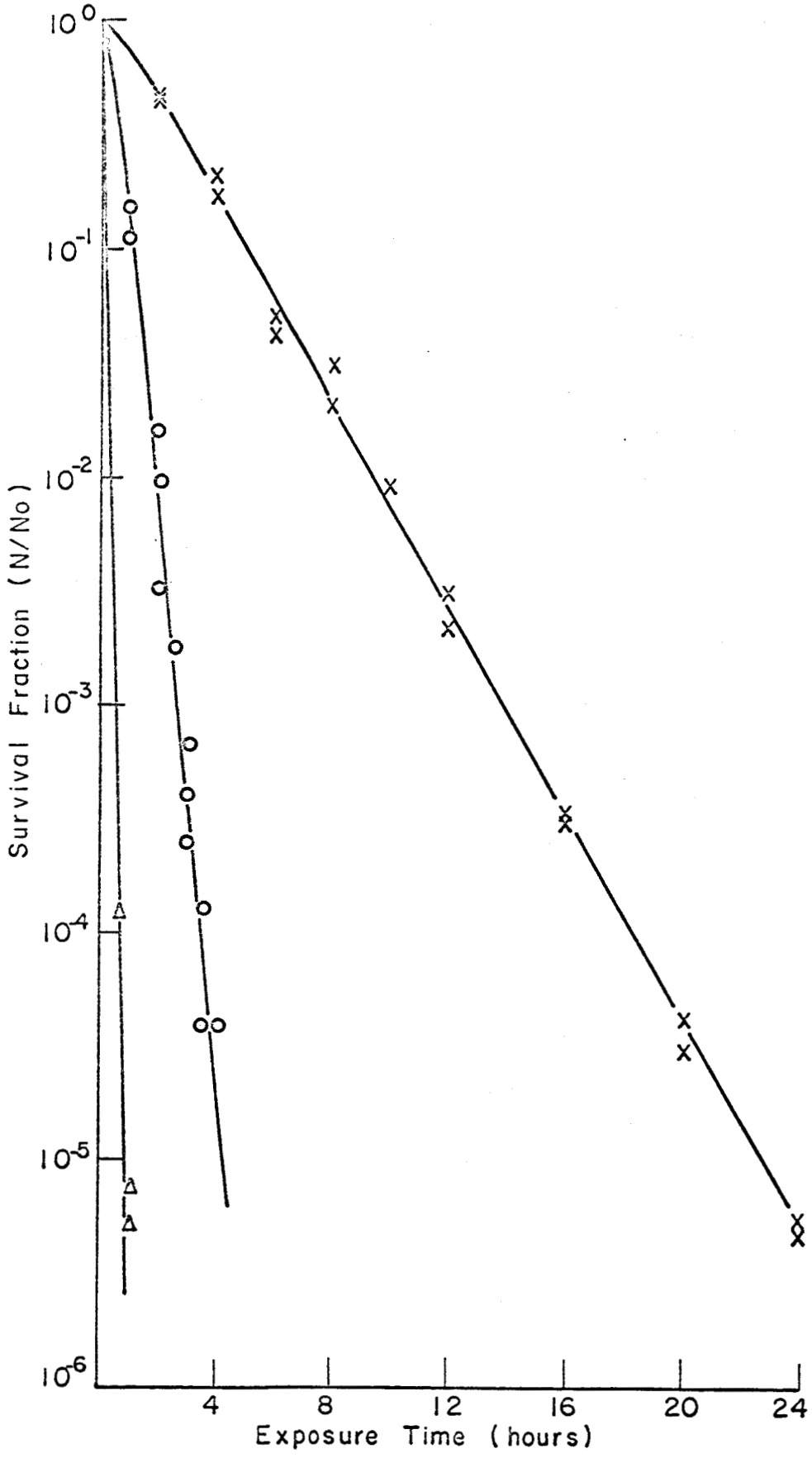

10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
Survival Fraction (N/No)
4
8
12
16
20
24
Exposure Time (hours)

Figure 4. Survival Curves for Air
For Bacillus subtilis var. niger in Oven #2

Key

Normal exposure at 120°C —×—×—

Exposure at 120°C with 30 seconds initial vacuum-forced convection —o—o—

Normal exposure at 120°C—oven #1 - - - - - -

Normal exposure at 135°C —Δ—Δ—

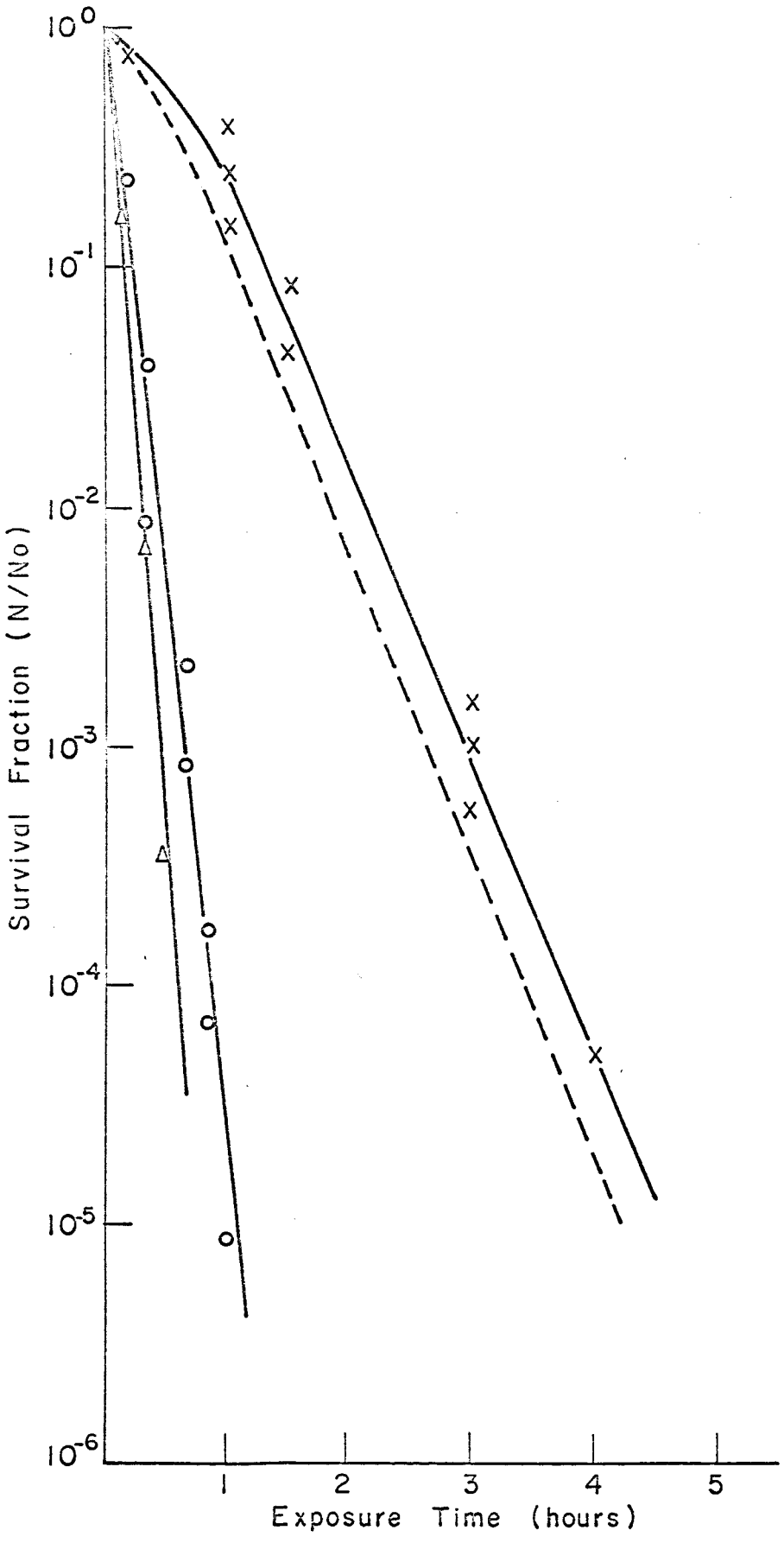
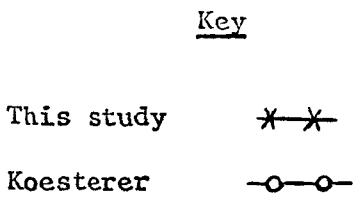

Survival Fraction (N/No)
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
1
2
3
4
5
Exposure Time (hours)

Figure 5. Arrhenius Curves for Bacillus subtilis var. niger
From Data in this Study
And Data from Studies of Koesterer (1964)



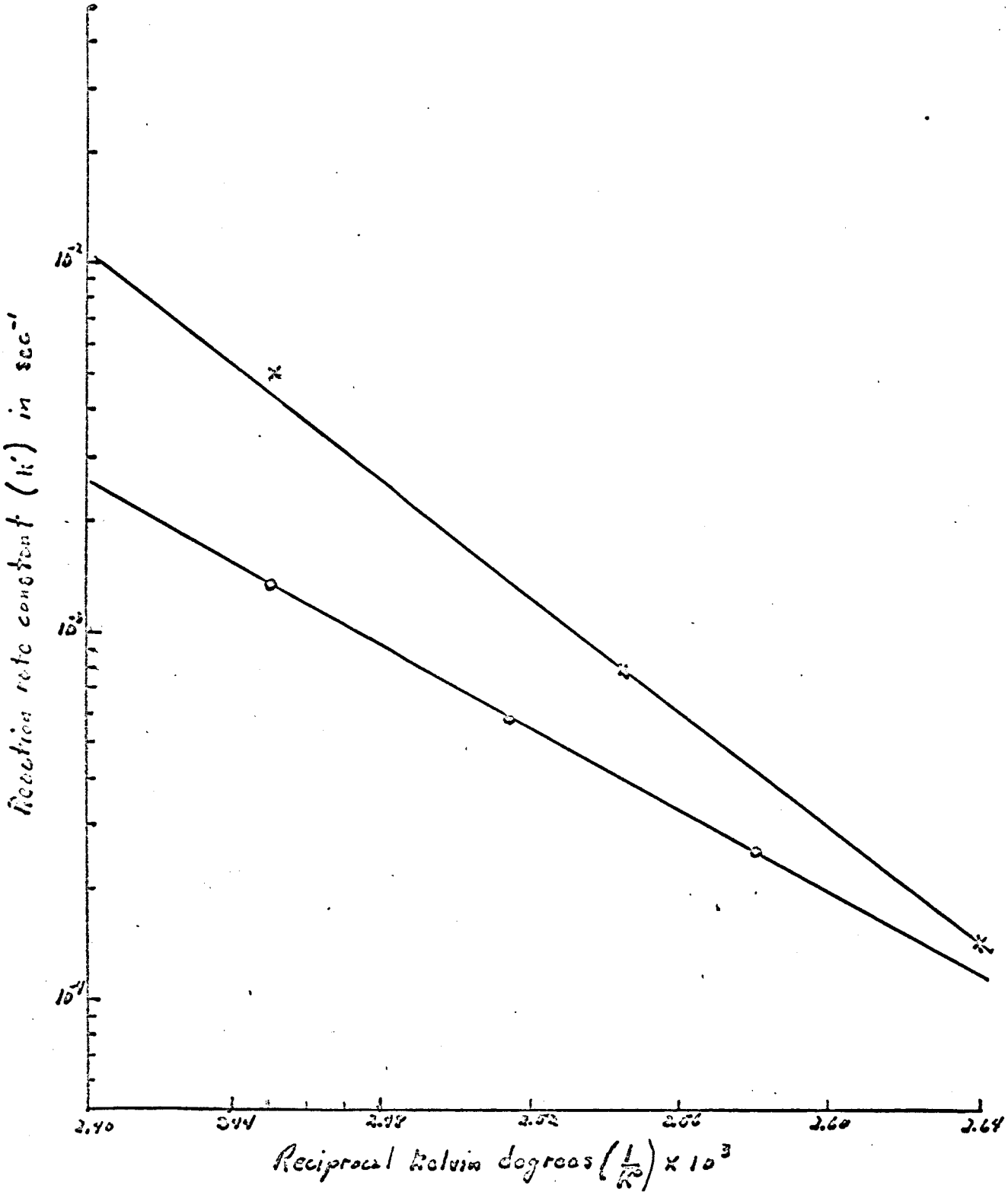
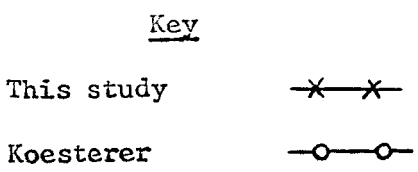
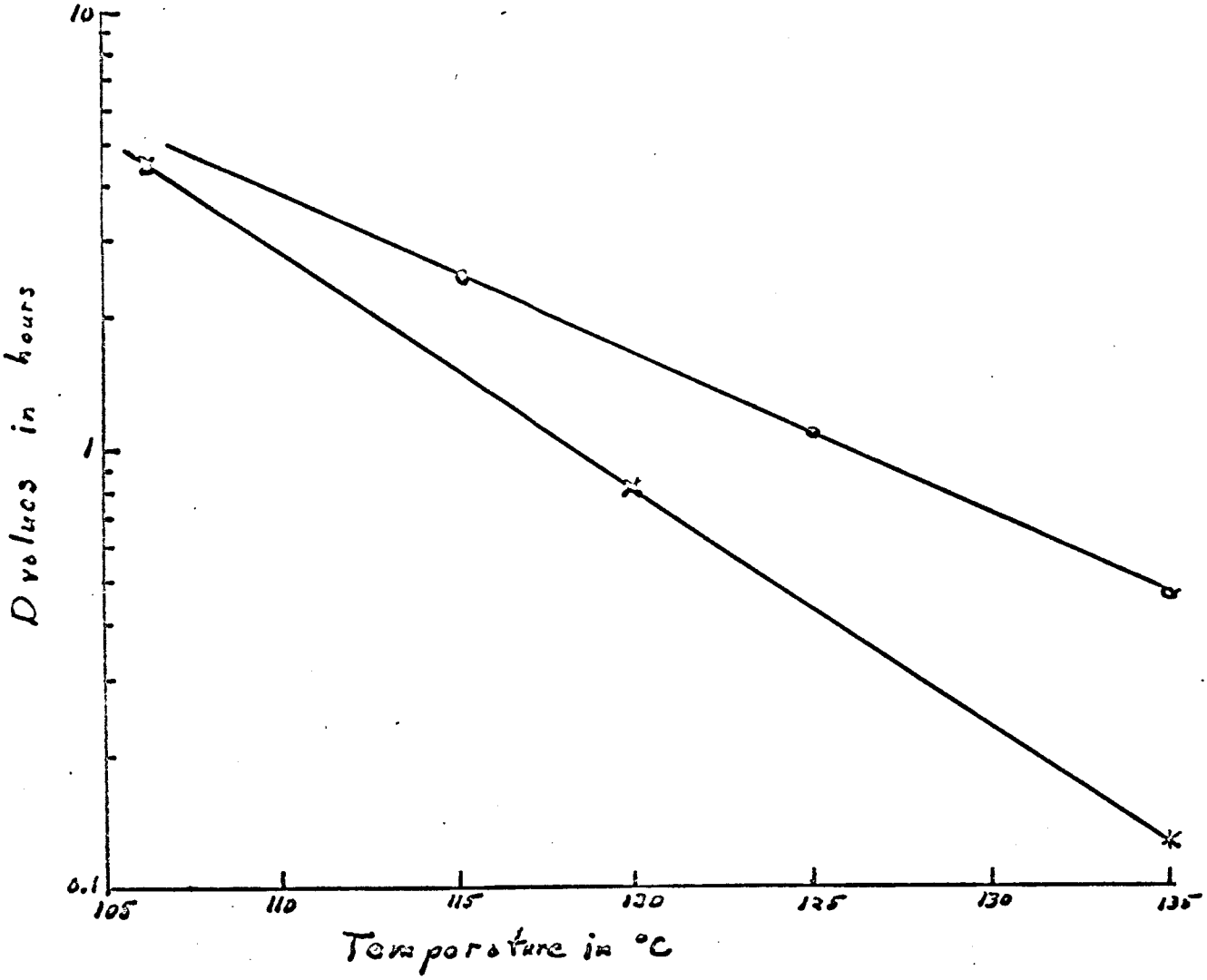

Reaction rate constant (k') in sec⁻¹
10⁻²
10⁻³
10⁻⁴
2.40
2.44
2.48
2.52
2.56
2.60
2.64
Reciprocal Kelvin degrees (1/T) × 10³

Figure 6. Phantom Thermal Death Time Curves
For *Bacillus subtilis* var. *niger* (D values versus T°C)
On Data from This Study
And Data from the Work of Koesterer (1964)




10
1
0.1
D values in hours
105
110
115
120
125
130
135
Temperature in °C

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