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Title: THERMALLY INDUCED LEAKAGE AND VIABILITY STUDIES IN AN OBLIGATE PSYCHROPHILIC MARINE BACTERIUM

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Abstract approved _____
Dr. R. Y. Morita

Vibrio marinus MP-1, an obligate psychrophilic marine bacterium, was severely damaged when heat-shocked in the presence of nutrients. Thermally induced leakage materials from cells tested for in the medium were 260 mμ absorbing material (nucleic acids), orcinol reacting material (RNA), ninhydrin reacting material (amino acids), protein, malic dehydrogenase, and glucose-6-phosphate dehydrogenase. Stationary phase cells were the most heat resistant as well as being more resistant to lysis and leakage after death. Log phase cells were the most thermolabile, and released intracellular materials after heating. There was an insignificant amount of leakage materials from cells held at 15 C (controls) while the 20, 23, and 25 C heat-shocked samples leaked increasingly more materials respectively. Leakage was shown to take place only after 95 percent of the cells were rendered nonviable. Leakage and lysis took place concommitantly.

Thermally Induced Leakage and Viability Studies in an Obligate Psychrophilic Marine Bacterium

by

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THERMALLY INDUCED LEAKAGE AND VIABILITY STUDIES IN AN OBLIGATE PSYCHROPHILIC MARINE BACTERIUM

INTRODUCTION

Previous investigations with Vibrio marinus MP-1, an obligate marine psychrophile, have shown that intracellular materials were released into the menstruum when the cells were heat-shocked. Protein, RNA, DNA, amino acids, malic dehydrogenase, and glucose-6-phosphate dehydrogenase were identified. These results posed the question: is thermal death due to a loss of cell membrane integrity? If the cell were unable to control what leaves and enters, death would likely ensue.

In previous studies, cells of V. marinus MP-1 were centrifuged and suspended in synthetic sea water before heat-shock. The ability of cells to withstand moderate temperature was greater when they were suspended in medium rather than in sea water or buffers. Since the presence of energy and nitrogen sources may make the cells less prone to leak, this investigation deals with the kinetics of leakage in the presence of nutrients as well as the kinetics of leakage in relation to the growth curve of the culture. In this thesis leakage will be considered to be the release of intracellular materials into the menstruum before or after lysis; and lysis, the dissolution of the cell but not necessarily the cell wall. Several parameters were used

as criteria for leakage: absorption at 260 mμ (nucleic acids), orcinol reacting material (RNA), protein, ninhydrin reacting material (amino acids), malic dehydrogenase, and glucose-6-phosphate dehydrogenase were determined in the suspending medium. Viability and turbidity studies were conducted to determine the relation between initiation of leakage, cell viability, and lysis.

LITERATURE SURVEY

The oceans, occupying over 70 percent of the earth's surface, provide a vast environment for the growth of psychrophilic (cold-loving) bacteria. The average temperature for the marine psychrosphere is approximately 4 C, and more than 90 percent is less than 5 C. Psychrophilic bacteria differ from mesophilic bacteria in that the former have a lower growth temperature. According to Stoke's definition (23), psychrophiles are able to grow rapidly enough at 0 C to form visible colonies within one week. Obligate psychrophiles have an optimum growth temperature below 20 C and facultative psychrophiles, above 20 C. ZoBell and Conn (28) showed that bacteria isolated from marine sediments were often thermosensitive to plating temperatures (45 C); also 45 percent were killed above 30 C within 10 min, and 80 percent were killed within 10 min at 40 C.

Numerous explanations have been proposed for causes of death above maximal growth temperatures in microorganisms. Among these are the coagulation of proteins, enzyme inactivation, melting of membrane lipids, degradation of RNA, leakage, and others.

Edwards and Rettger (4) suggested the denaturation of proteins, especially enzymes, as one of the factors causing death

above maximal growth temperatures in mesophilic bacteria. They found excellent agreement between maximal growth temperatures and denaturation of several respiratory enzymes in Bacillus spp. Hagen and Rose (6) showed that the inactivation of one or more TCA respiratory enzymes limited the growth of a psychrophilic yeast, Cryptococcus sp. Upadhyay and Stokes (25) demonstrated formic dehydrogenlyase to be more heat labile in a facultative psychrophilic strain of Escherichia coli than in an obligate mesophilic strain. The enzyme was most active at 30 C in the facultative strain, and inactivated at 45 C, while in the mesophilic strain it was most active at 45 C and inactivated at 70 C. Langridge and Morita (13) found malic dehydrogenase to be inactivated at 20 C in V. marinus MP-1. This obligate psychrophilic marine bacterium has a maximal growth temperature of 20 C (16). Burton and Morita (2) demonstrated malic dehydrogenase to be inactivated at 30 C in V. marinus PS-207, the maximal growth temperature for this facultative psychrophile.

The synthesis or accumulation of metabolic poisons may cause death at elevated temperatures where a temperature sensitive enzyme may cause the accumulation of an intermediate which inhibits other enzymes (19). Hagen and Rose (6) suggested an increased use of the intracellular amino acid pool at elevated temperatures. They found a rapid decrease in the intracellular amino acid pool in a Cryptococcus yeast when heat-shocked. The disruption of

intracellular organization may cause death in psychrophiles because data indicated differences in cellular organization rather than enzymatic differences with mesophiles (11). Wood (27) suggested that death from heat may be a result of a detrimental alteration in the ability of bacterial cells to reproduce.

In 1931 Bělehrádek (1) proposed the "lipid liberation theory" whereby membrane lipids melt causing death above maximal growth temperatures. The greater the saturation of fatty acids, the greater the melting point. The amount of unsaturated fatty acids increased in cellular lipids in E. coli when shifted to lower temperatures (14, 21). Kates and Hagen (12) found the degree of saturation to decrease in Serratia marcescens grown at 10 C over those at 30 C.

Heilbrunn (10) discussed the possibility of death in animal cells by calcium coagulating cellular materials. Calcium, bound to membrane phospholipids, may be liberated upon melting of the membrane lipids.

Staphylococcus aureus, Bacillus subtilus, and E. coli released RNA when heat shocked (3), with more from lag and log phase cells than from later phases. This process did not depend upon the denaturation of proteins because it occurred at lower temperatures. Similar results occurred with Aerobacter aerogenes (24). A relationship was indicated between rates of death and RNA degradation. Most experiments showed a maximum rate of leakage, indicated by

increased extinctions at 225 mμ and increased ninhydrin reacting materials in the menstruum, before a significant loss of viability could be detected. This indicated leakage, indicative of RNA breakdown, took place before death.

Evison and Rose (5) were unable to show a notable increase in 260 mμ absorbing material in the supernatant fluid when Candida was heat shocked for 48 hr at 25 C, 5 C above its maximal growth temperature. Slight leakage was detected in Arthrobacter after 48 hr when transferred to 37 C, 5 C above the maximal growth temperature.

The maximal growth temperature for an obligate marine psychrophile isolated from flounder eggs was 21 C, and above this temperature death readily ensued (7). Hagen, Kushner and Gibbons (7) believed death was probably not due to cell wall or membrane breakdown because 90 percent of the cells were dead before there was a decrease in turbidity. As the turbidity decreased, 260 mμ absorbing material increased in the supernatant. These data would indicate that leakage followed lysis, and lysis followed death.

Burton and Morita (2) and Morita and Burton (15) demonstrated that a loss of membrane permeability may cause death in V. marinus PS-207. Malic dehydrogenase was much more sensitive to heat with lysed cells.

Robison and Morita (18) found V. marinus MP-1 to shrink in

size and release material with an absorption peak at about 260 mμ when heated above the maximal growth temperature. They found the absorbance to be lowest at 15 C with pronounced increases above 20 C, and a radical increase above 28 C. Haight and Morita (8) showed greater leakage resulted from 4 C than 15 C grown cells of V. marinus MP-1.

Haight and Morita (9) elaborated on the leakage phenemonon in V. marinus MP-1 harvested during the log growth phase. Of the leakage products analyzed, the order of leakage was protein, RNA, DNA, and lastly amino acids. Polymeric and nonpolymeric ribonucleic acids were found in the menstruum. At 29.7 C leakage was initiated after 15 min. Very little leakage material was detected when cells were heated at the growth optimum, 15 C, for 60 min.

MATERIALS AND METHODS

Growth Media

SDB medium was prepared by adding the following to 1,000 ml distilled water: Rila marine salts (Rila Products, Teaneck, N. J.), 5.0 g; NaCl, 15 g; glucose, 0.5 g; succinic acid, 0.2 g; polypeptone (BBL), 5.0 g; yeast extract (Difco), 3.0 g; ferric sulfate, 0.005 g. The pH was adjusted to 7.5 with NaOH, and autoclaved at 15 psi for 20 min. The pH after autoclaving was 7.4. SDB agar was prepared with 1.5 percent agar (Difco) added to SDB medium.

SOS medium was prepared by adding the following to 1,000 ml distilled water: succinic acid, 2.5 g; $(NH_4)_2SO_4$, 2.0 g; $K_2HPO_4 \cdot 3H_2O$, 5.2 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; NaCl, 20.4 g; vitamin solution, 10.0 ml; trace element solution, 10.0 ml. The vitamin solution was prepared by adding the following to 1,000 ml distilled water: nicotinamide, 400 mg; thiamine, 100 mg; pyridoxine, 100 mg; Ca-D-pantothenate, 100 mg; riboflavin, 25 mg; biotin, 1 mg. Trace element solution was prepared by adding the following to 1,000 ml distilled water: $FeCl_3 \cdot 6H_2O$, 100 mg; $CaCl_3 \cdot 2H_2O$, 200 mg; NaBr, 100 mg; $MnCl_2 \cdot 4H_2O$, 100 mg.

All media were cooled to 15 C before inoculation.

Organism

Vibrio marinus MP-1 (ATCC 15381), isolated by Morita and Haight (16) was used in these studies. Stock cultures were maintained in SDB and SOS broth in screw cap test tubes at 5 C and transferred monthly.

Growth of Cells

Five ml from a stock culture was inoculated into 50 ml of the same medium in a 250 ml Erlenmeyer flask and incubated at 15 C for 12 hr in an incubator shaker (New Brunswick Psycho-Term) with a reciprocating mechanism set at 120 strokes per minute and a 2.54 cm stroke. Thirty ml of this culture was then inoculated into 300 ml of the same medium and allowed to incubate for 12 hr as before. From this culture 50 ml was inoculated into 500 ml of the same medium in a Fernbach flask and incubated in a similar manner. The latter culture was used for all studies.

Growth curves were determined in both SDB and SOS media after two 12 hr transfers. Optical densities were read at 425 mμ in a Spectronic 20 colorimeter in 12 x 112 mm tubes (Bausch and Lomb) with a medium blank at 60 min intervals.

Where cells were to be concentrated, they were centrifuged in sterile 250 ml polypropylene centrifuge containers at 6,000 x g for

10 min at 15 C in a Sorvall RC-2 superspeed centrifuge. The supernatant was decanted and the pellet resuspended in fresh medium.

Heat-Shocking

SDB grown cells were concentrated so that a 1:100 dilution in medium gave an optical density of 0.35 at 425 mμ in a Spectronic 20 colorimeter with a medium blank. Sixty ml were placed aseptically in sterile 250 ml Erlenmeyer flasks and allowed to stand at 15 C for 90 min before placing in water baths with no agitation at either 20, 23, or 25 C. A 15 C control was kept in a 15 C constant temperature incubator. Samples were taken at 0, 20, 45, 60 or 65, 90, and 120 min after agitating manually to insure even suspensions. Viability was estimated as described under Methods of Enumeration, and lysis estimated as a decrease in optical density at 425 mμ. Five ml of each heat-shocked sample was centrifuged in polypropylene centrifuge tubes at 27,000 x g for 5 min at 15 C. Supernatants were decanted and stored in a freezer at -20 C until analyzed.

To determine relative leakage during the growth curve in SDB medium, cells were concentrated at various times during the growth curve so a 1:100 dilution in medium gave an optical density of 0.15 at 425 mμ. Five ml was placed in each of two polypropylene centrifuge tubes; one was placed in a 25 C water bath and the control in a 15 C constant temperature incubator for 120 min. The cells

were centrifuged at 27,000 x g for 5 min at 15 C, and supernatants collected. The difference in absorbance at 260 mμ between the 15 C control and the 25 C heat-shocked sample was determined.

To estimate the percent survival at different times during the growth curve in SDB medium, 50 ml of a culture was placed aseptically, at different times, in sterile 250 ml Erlenmeyer flasks without concentration. One was placed in a 25 C water bath and the control in a 15 C constant temperature incubator. Agitation was provided only before samples were withdrawn to provide an even suspension. Viability was estimated as described under Methods of Enumeration, and the percent remaining viability calculated.

Heat-shocking in SOS medium was carried out directly without concentrating the cells. Fifty ml of the culture was placed into two sterile 250 ml Erlenmeyer flasks, one placed in a 25 C water bath, and the control in a 15 C constant temperature incubator. There was no agitation except before samples were withdrawn.

Methods of Enumeration

One ml of a cell suspension was pipetted into a chilled 9 ml sea water dilution blank in a screw cap test tube. Rila marine salts (Teaneck, N. J.), 35 g per liter adjusted to pH 7.5, were used. The tube was shaken vigorously 25 times and 1 ml pipetted serially into chilled 9 ml dilution blanks. Three spread plates were made for

each dilution tested using chilled SDB agar plates. Plates were incubated at 15 C for 72 hr and the mean number of colonies for the 3 plates computed.

Analysis of 260 mμ Absorbing Material

Materials absorbing at 260 mμ in the supernatants from SDB heat-shocked cells were estimated in a Beckman DB spectrophotometer in a quartz cuvette with a 1 cm path length using a distilled water blank. The supernatants were diluted 1:100 with distilled water. Supernatants from SOS heat-shocked cells were read directly, with no dilution necessary.

Orcinol Reacting Material

Orcinol reacting material was assayed by a modification of Schneider's (20) technique. The reagent was prepared by adding 0.10 g $FeCl_6 \cdot 6H_2O$ and 0.10 g Orcinol (Sigma) to 100 ml concentrated HCl, and the standard curve prepared with purified yeast RNA (Sigma) from 0 to 100 μg per ml, in 0.5 N NaOH. One ml of properly diluted sample was added to a test tube with 3 ml of orcinol reagent, and steamed for 15 to 20 min. The tubes were allowed to cool and the optical density read at 660 mμ in a Beckman DB spectrophotometer against a reagent blank.

Protein Determination

Protein was estimated by the method of Warburg and Christian (26).

Amino Acid Assay

Amino acids were analyzed colorimetrically by a modification of the ninhydrin technique as described by Spies (22). The ninhydrin reagent was prepared by dissolving 5.0 g ninhydrin (1, 2, 3-triketohydrindene, Eastman Organic Chemicals) in 500 ml 0.2 N citrate buffer (42.0 g citric acid monohydrate plus 400 ml 1.0 N NaOH to 1 liter). This was added to 500 ml citrate buffer in which 0.80 g reagent grade $SnCl_2 \cdot 2H_2O$ had been dissolved. The precipitate which resulted from mixing the two solutions was removed by filtering through Whatman No. 2 filter paper in a Büchner funnel.

The standard curve was prepared with glycine from 0.3 to 1.5 μmoles per ml. One ml of properly diluted sample was pipetted into a test tube and 1 ml of ninhydrin reagent added. Tubes were steamed for 15 min, and 2 ml diluent (equal volumes n-propanol and distilled water) added while hot. After 15 min the optical density was measured at 570 mμ in a Beckman DB spectrophotometer.

Glucose-6-Phosphate Dehydrogenase Assay

Glucose-6-phosphate dehydrogenase activity was estimated by the change in optical density at 605 mμ as 2, 6-dichlorophenolindophenol was reduced. The reaction mixture contained the following: 0.10 ml dichlorophenolindophenol, 0.05 percent; 0.10 ml glucose-6-phosphate, 10 μmoles per ml; 0.10 ml TPN, 0.10 μmoles per ml; 0.10 ml phenazine metasulfate, 1.0 mg per ml; 1.00 ml Tris-SO_4, pH 8.0, 520 μmoles per ml; 1.40 ml distilled water; and 0.20 ml supernatant. Change in optical density per min was determined against a reagent blank with distilled water in place of supernatant. A Beckman DU spectrophotometer was used fitted with a thermospacer connected to a circulating water bath at 20 C.

Malic Dehydrogenase Assay

The assay method measured the oxidation of reduced nicotinamide adenine dinucleotide (NADH) at 340 mμ as described by Ochoa (17). The decrease in optical density was measured in a Beckman DU spectrophotometer fitted with a thermospacer connected to a circulating water bath at 20 C. The reaction mixture contained in 3.00 ml; 1.6 μmoles oxaloacetic acid; 0.2 μmoles NADH; 520 μmoles Tris-HCl, pH 7.4; and 0.20 ml of supernatant to be assayed.

Photomicrographs

Photomicrographs were taken with a Leitz Ortholux microscope equipped with a Heine phase contrast condensor #74 and Pv Fl 70/1.15 n objective. Photographs were taken with the use of a green filter, Kodak High Contrast Copy film, and Olympus PM-6 camera attachment.

RESULTS AND DISCUSSION

Preliminary leakage studies in SDB medium were unpredictable, with often no detectable leakage. This was remedied by rigorously standardizing cell harvest times with the growth curve (Figure 1); optical densities were compared to the growth curve. When cells were harvested, concentrated, and heat-shocked during the log phase, leakage always resulted (Figure 1). However, when cells were harvested, concentrated, and heat-shocked after approximately 7 hr of growth, approaching or during the stationary phase, there was a sudden and marked decrease in leakage. After this time little or no leakage could be detected.

Cells were more resistant to thermally induced death in older cultures. When cells were heat-shocked at 25 C in SDB medium for 90 min, the percent survival increased markedly as a culture approached the stationary phase (Figure 1). Figure 2 illustrates loss of viability of early log phase cells as a function of the duration of heat-shock. Figures 3 and 4 illustrate loss of viability of mid-log phase cells and negative growth acceleration phase cells respectively. After 90 min heat-shock at 25 C, 1 percent of the early log phase cells remained viable (85×10^6 cells per ml killed); 11 percent of the cells from the middle of the log phase remained viable (300×10^6 cells per ml killed); and 40 percent remained viable (150×10^7

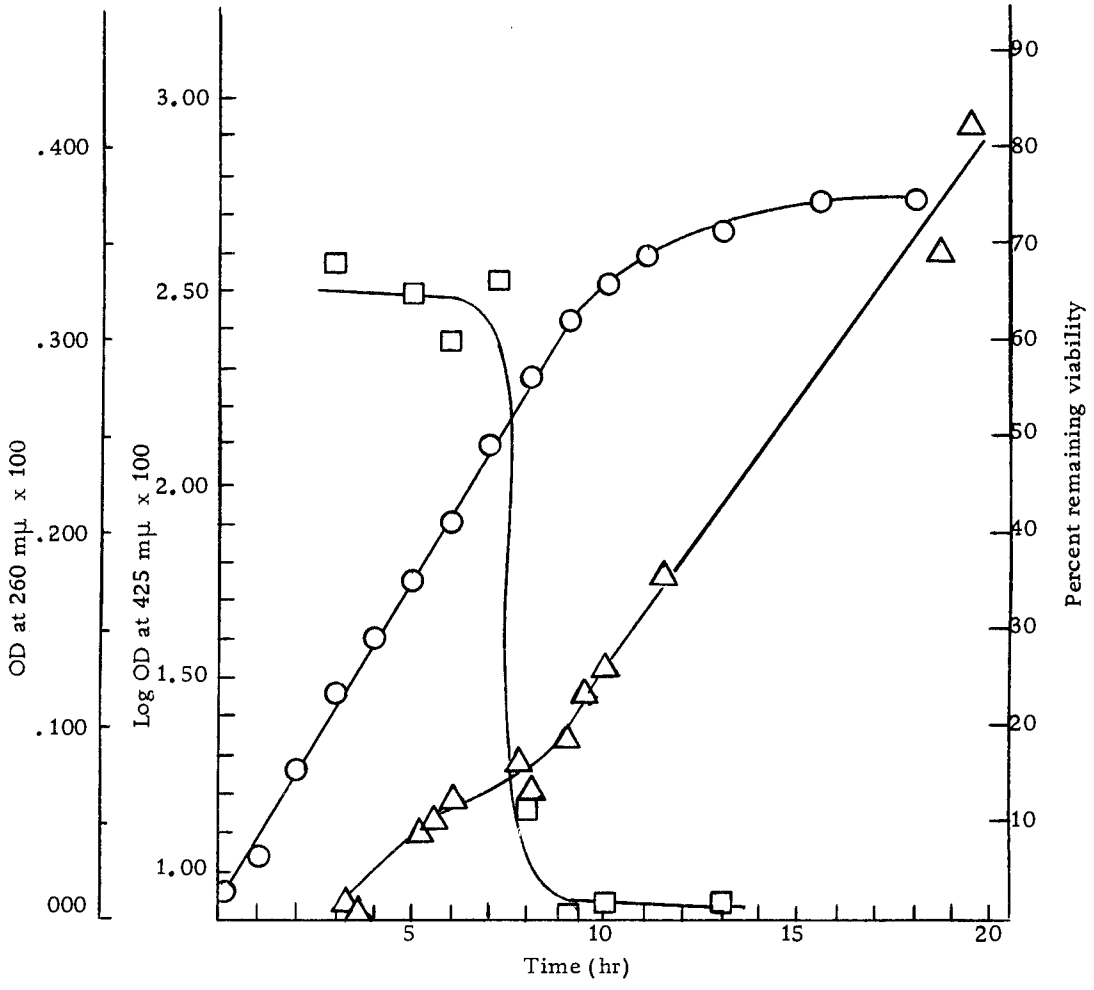

Figure 1. Leakage and viability in relation to the growth curve (○) of V. marinus cells in SDB medium. For leakage studies, cells were taken at various times during the growth curve, concentrated, and heat-shocked at 25 C (□). Cells were heated directly without concentration at 25 C for 90 min at various times of the growth curve and the percent remaining viability estimated (△).

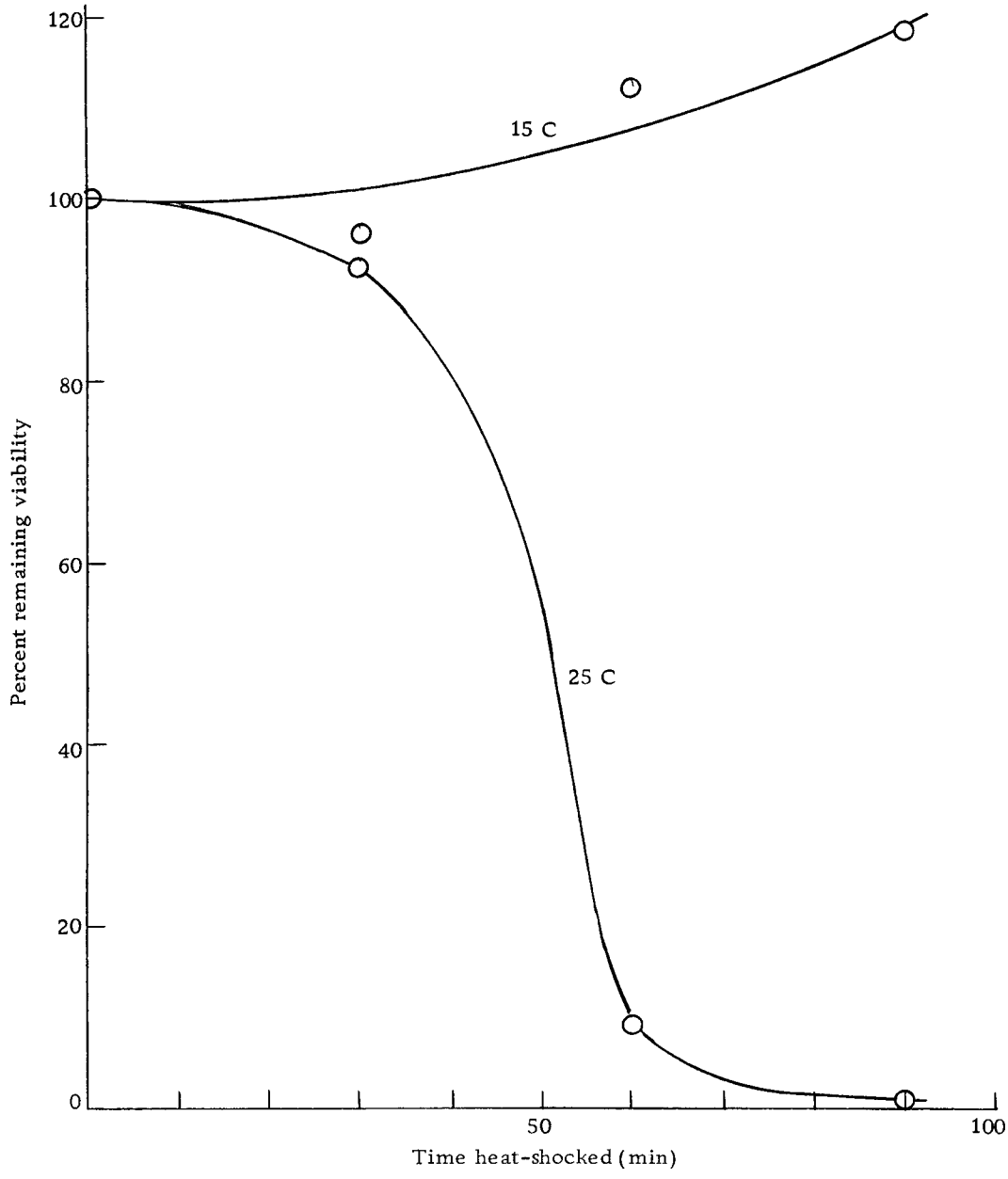

Figure 2. The percent survival of V. marinus cells heat-shocked in SDB medium at 25 C in relation to time. The cells were obtained from the early part of the log phase of growth. The one hundred percent level is equivalent to 85 x 10^6 cells per ml.

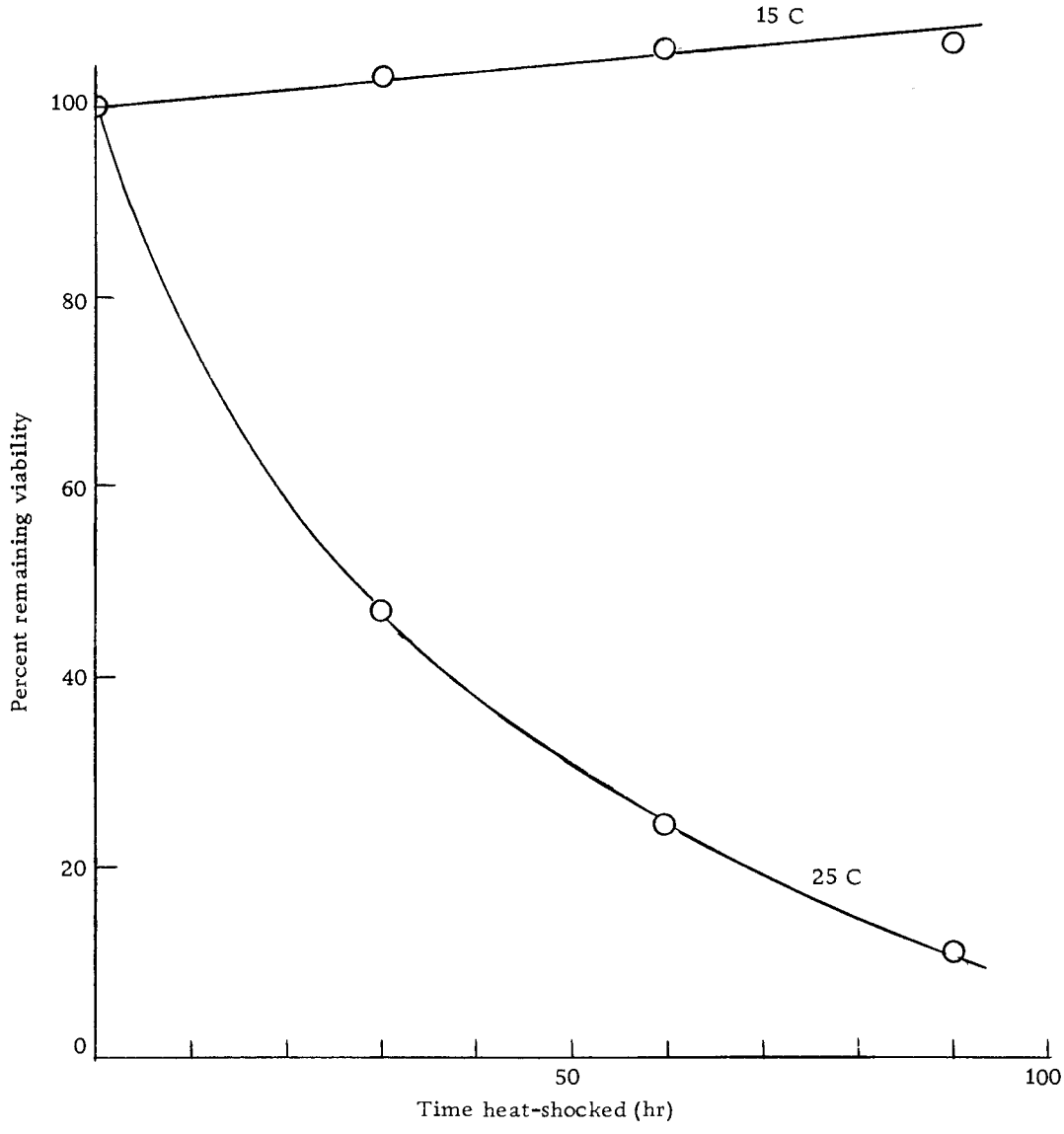

Figure 3. The percent survival of V. marinus cells heat-shocked in SDB medium at 25 C in relation to time. The cells were obtained from the middle of the log phase of growth. The one hundred percent level is equivalent to 300×10^6 cells per ml.

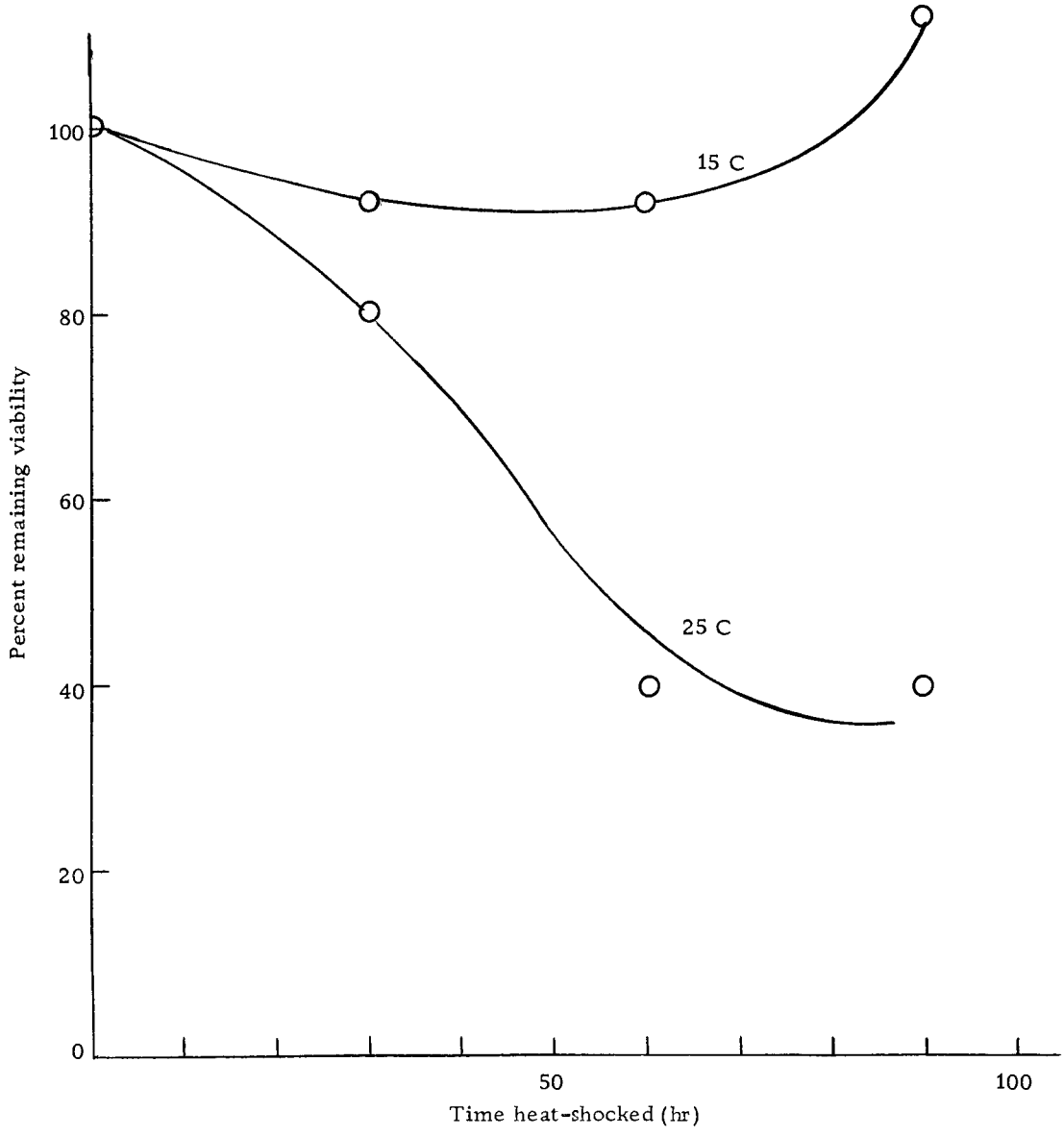

Figure 4. The percent survival of V. marinus cells heat-shocked in SDB medium at 25 C in relation to time. The cells were obtained from the negative growth acceleration phase. The one hundred percent level is equivalent to 150×10^7 cells per ml.

cells per ml killed) from the negative growth acceleration phase. It is evident that the cells in an older culture were far more resistant to thermally induced death, but more actually died.

This increased resistance of an older culture to heat could be due to factors other than increased sensitivity normally attributed to actively metabolizing log phase cells. The increased resistance might be due to an alteration of the culture medium during growth. As the culture grows, nutrients are removed from the medium and natural leakage and lysis products added. Also, after heat-shock, thermally induced leakage products might afford protection for the remaining viable cells. The data shown in Figures 5 and 6 indicate that such is not the case. Cells in both experiments were resuspended in fresh SDB medium before heat-shocking at 25 C; those in Figure 5 were in the log phase, and Figure 6 in the stationary phase. Figure 5 indicates that 95 percent of the log phase cells were killed within 20 min at 25 C. Within 25 min from this time (45 min total heat-shock), there were fewer than 0.2 percent remaining viable cells, with a marked increase in leakage over the 15 C control. A period of 55 minutes was required to kill 95 percent of the stationary phase cells. Sixty-five minutes later (120 min total heat-shock), there was less than 0.1 percent of the cells remaining viable. There was not a significant increase in leakage over the 15 C control. It would appear that cells in the stationary phase were more heat

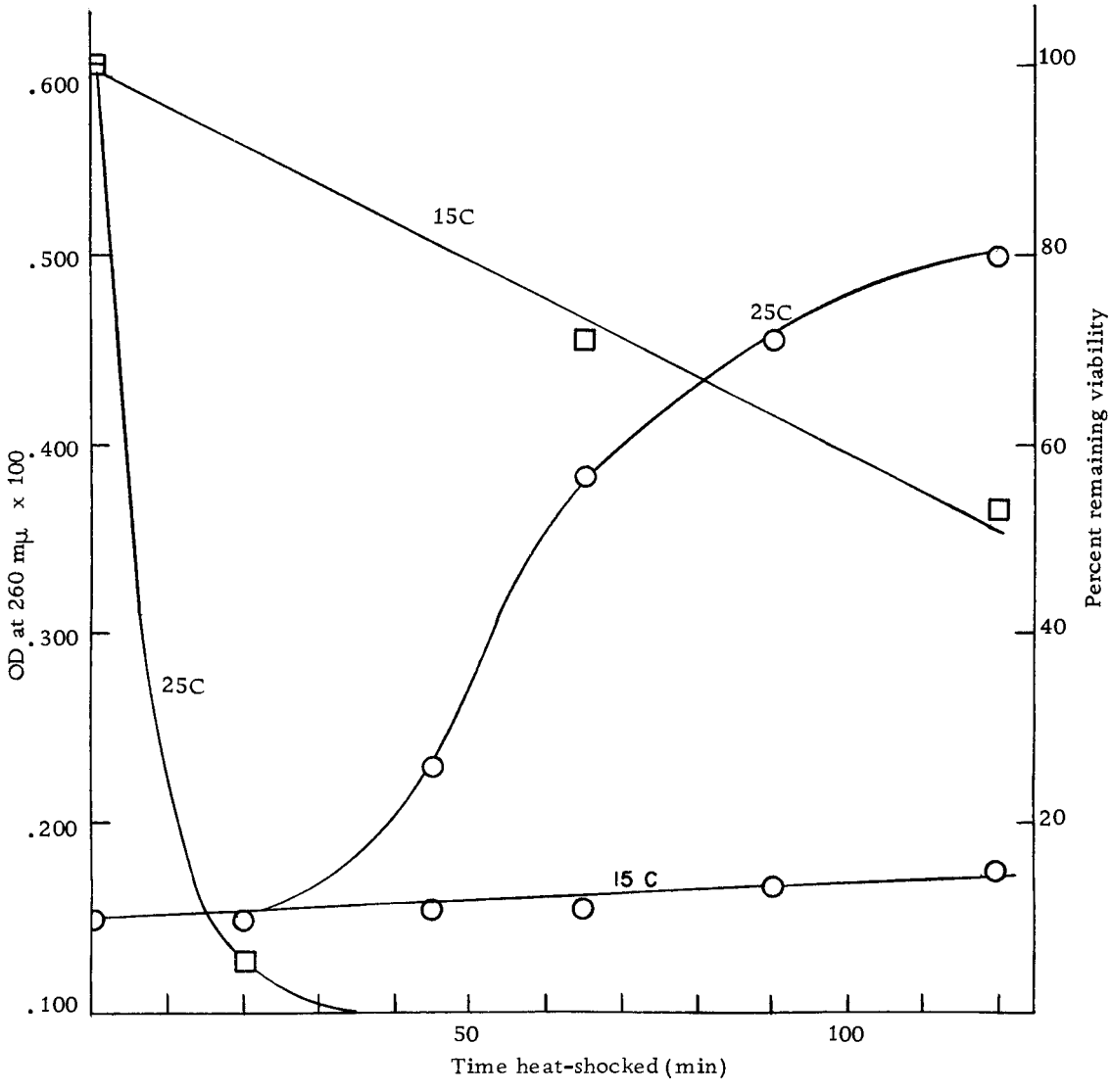

Figure 5. Viability (□) and leakage (○) as a function of time when log phase cells were heat-shocked in SDB medium at 25 C. One hundred percent viability is equivalent to 85 x 10^8 cells per ml.

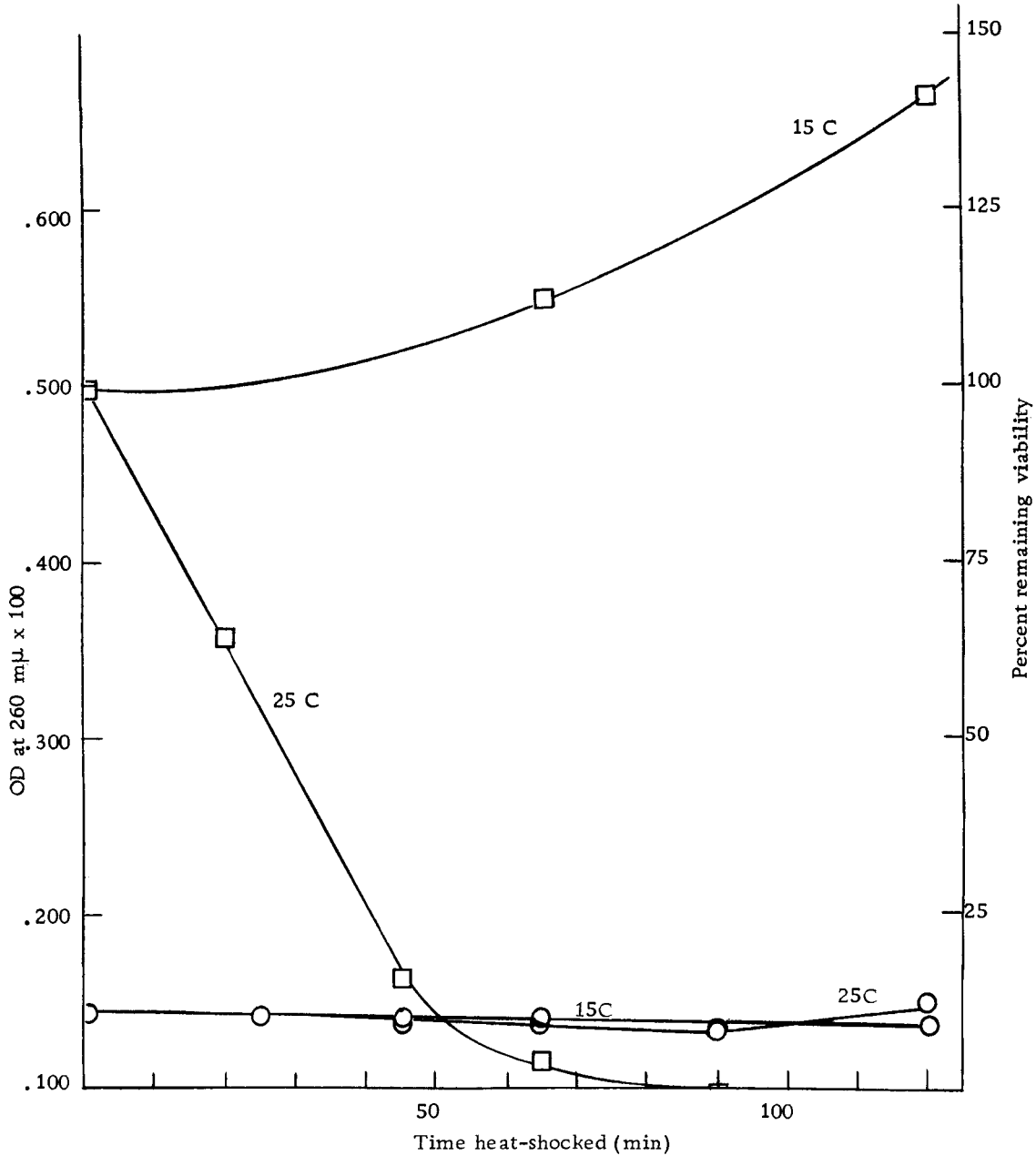

Figure 6. Viability (□) and leakage (○) as a function of time when stationary phase cells were heat-shocked in SDB medium at 25 C. One hundred percent viability is equivalent to 17×10^9 cells per ml.

resistant, and once thermal death had been evoked, it took much longer for these cells to leak than log phase cells. Thermal resistance would, therefore, not seem to be due to an alteration in the medium.

The most extensive leakage studies with V. marinus MP-1 were done by Haight (9) at this laboratory. In his investigation cells were concentrated and suspended in synthetic sea water. He showed the leakage of protein, RNA, DNA, and amino acids. Polymeric and nonpolymeric ribonucleic acids were demonstrated. Haight's (9) work indicated, by the increase of these materials in the menstruum, that cells were severely damaged when heat-shocked in the absence of nutrients. His work did not show the relation between leakage, lysis, and viability.

The data in this investigation shows that V. marinus MP-1 was also severely damaged when heat-shocked in the presence of nutrients. Cells leaked 260 mμ absorbing material (nucleic acids), orcinol reacting material (RNA), ninhydrin reacting material (amino acids), protein, malic dehydrogenase, and glucose-6-phosphate dehydrogenase increasingly at higher temperatures (Figures 7 through 12 respectively). There was no significant leakage from the 15 C controls while the 20, 23, and 25 C heat-shocked samples leaked increasingly. Quite likely many other soluble intracellular materials were also released.

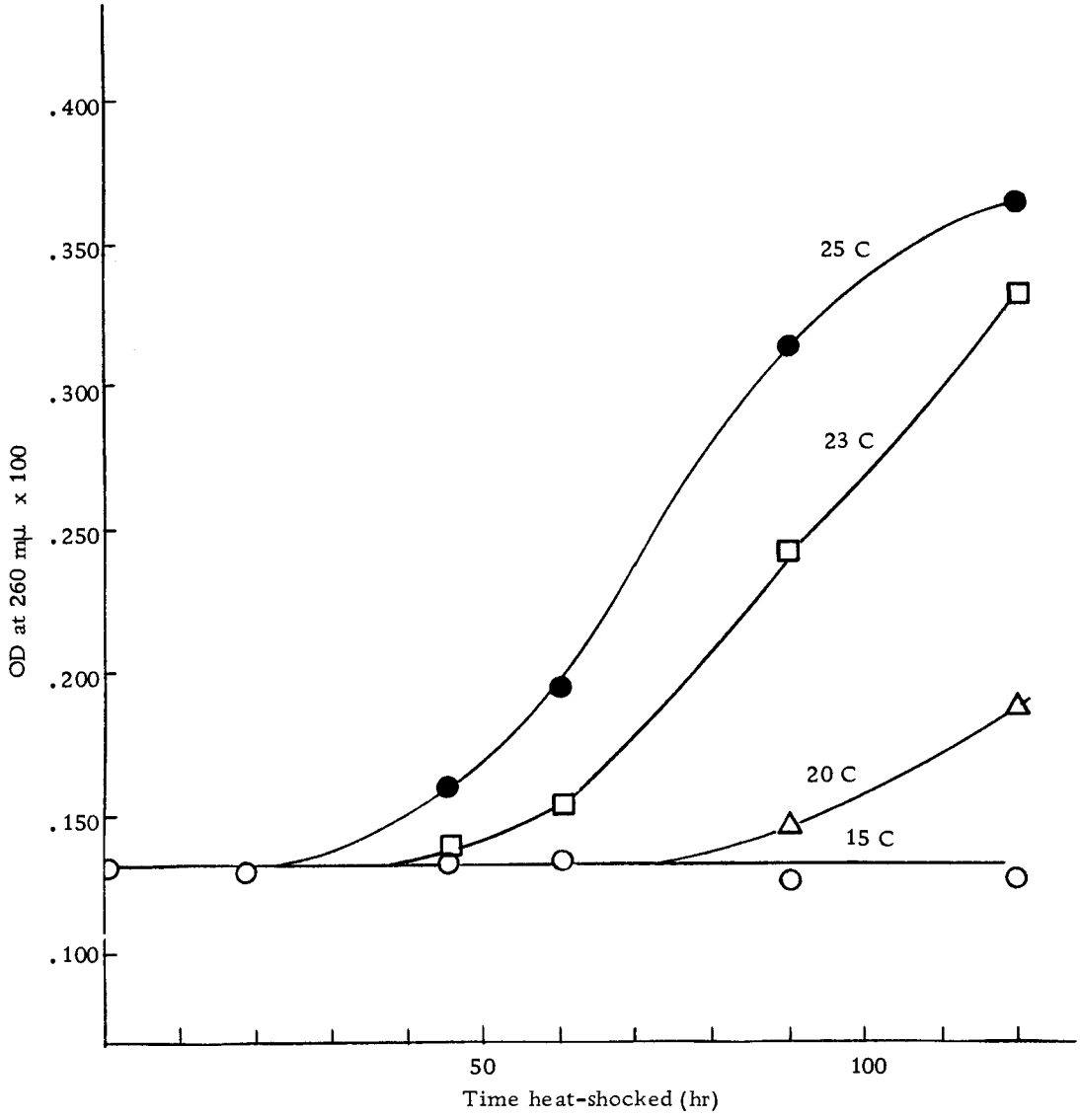

Figure 7. Leakage of 260 mμ absorbing material from V. marinus cells (obtained from the log phase) when heat-shocked at various temperatures in SDB medium in relation to time.

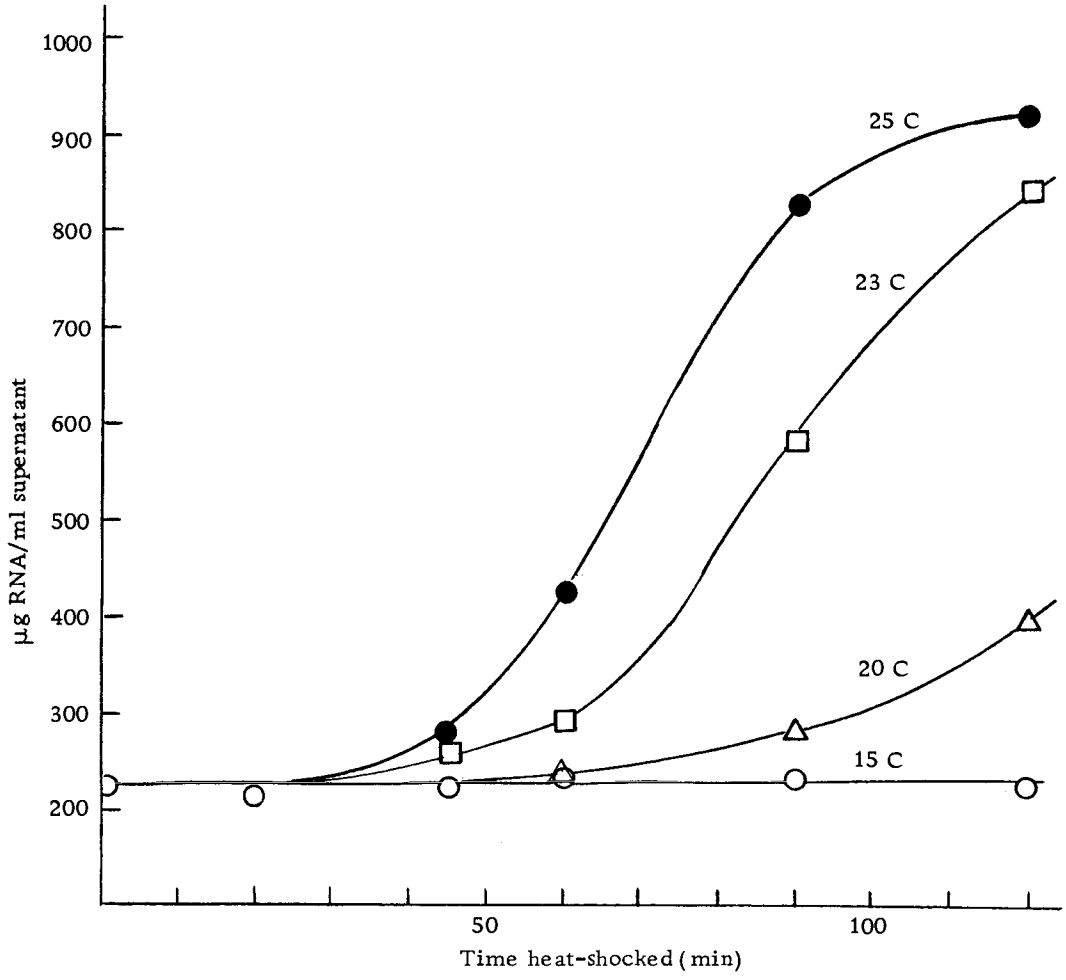

Figure 8. Leakage of orcinol reacting material from V. marinus cells (obtained from the log phase) when heat-shocked at various temperatures in SDB medium in relation to time.

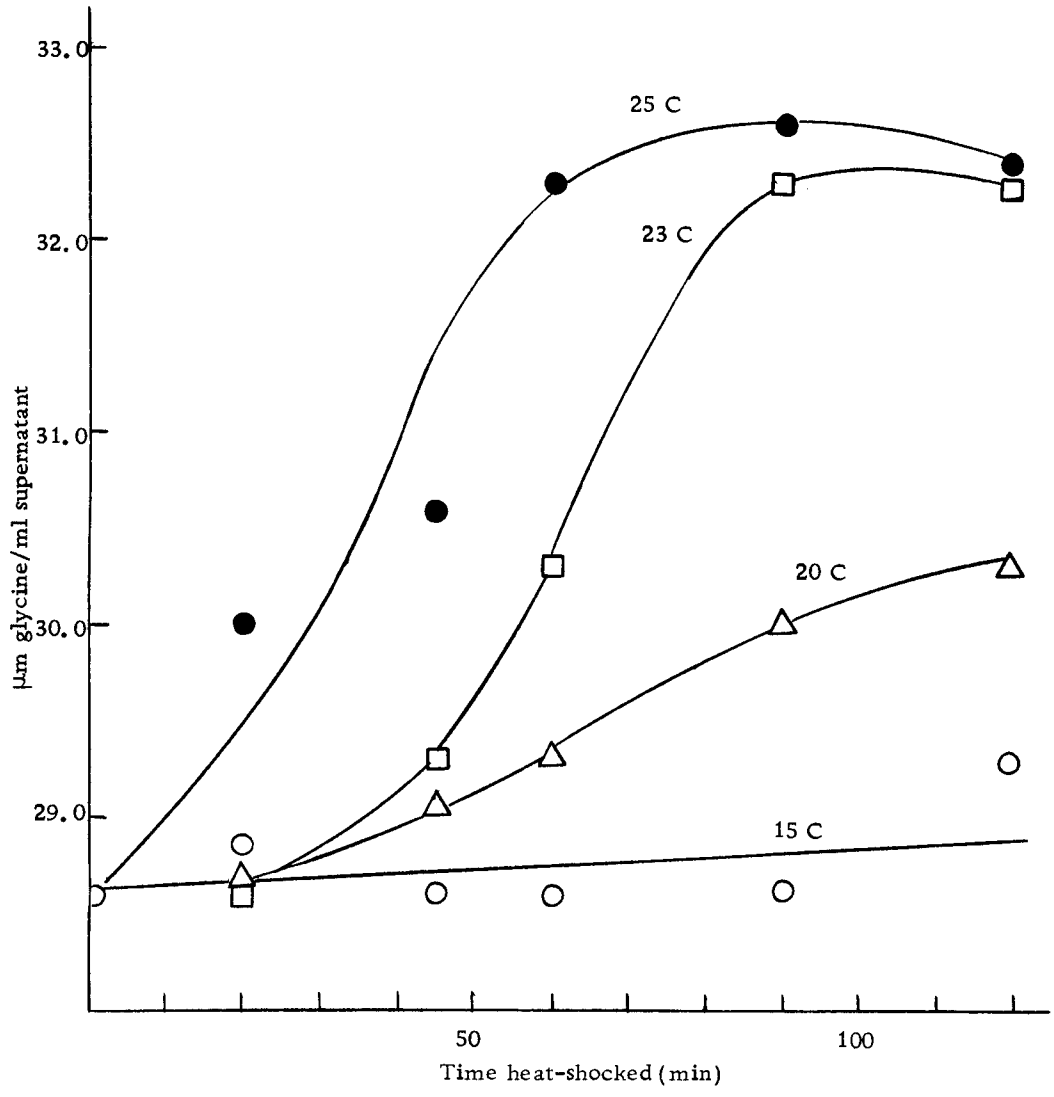

Figure 9. Leakage of ninhydrin reacting material from V. marinus cells (obtained from the log phase) when heat-shocked at various temperatures in SDB medium in relation to time.

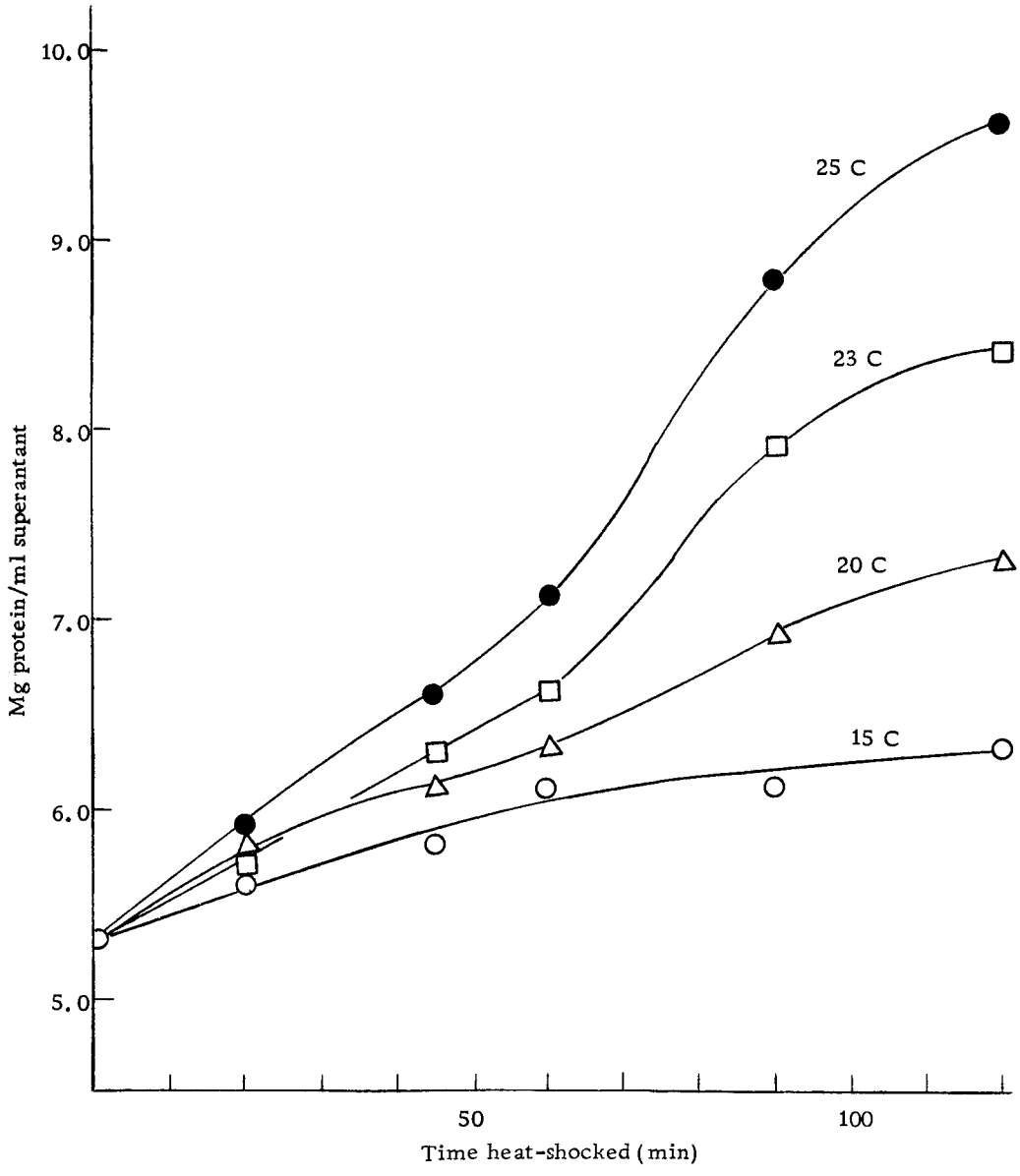

Figure 10. Leakage of protein from V. marinus cells (obtained from the log phase) when heat-shocked at various temperatures in SDB medium in relation to time.

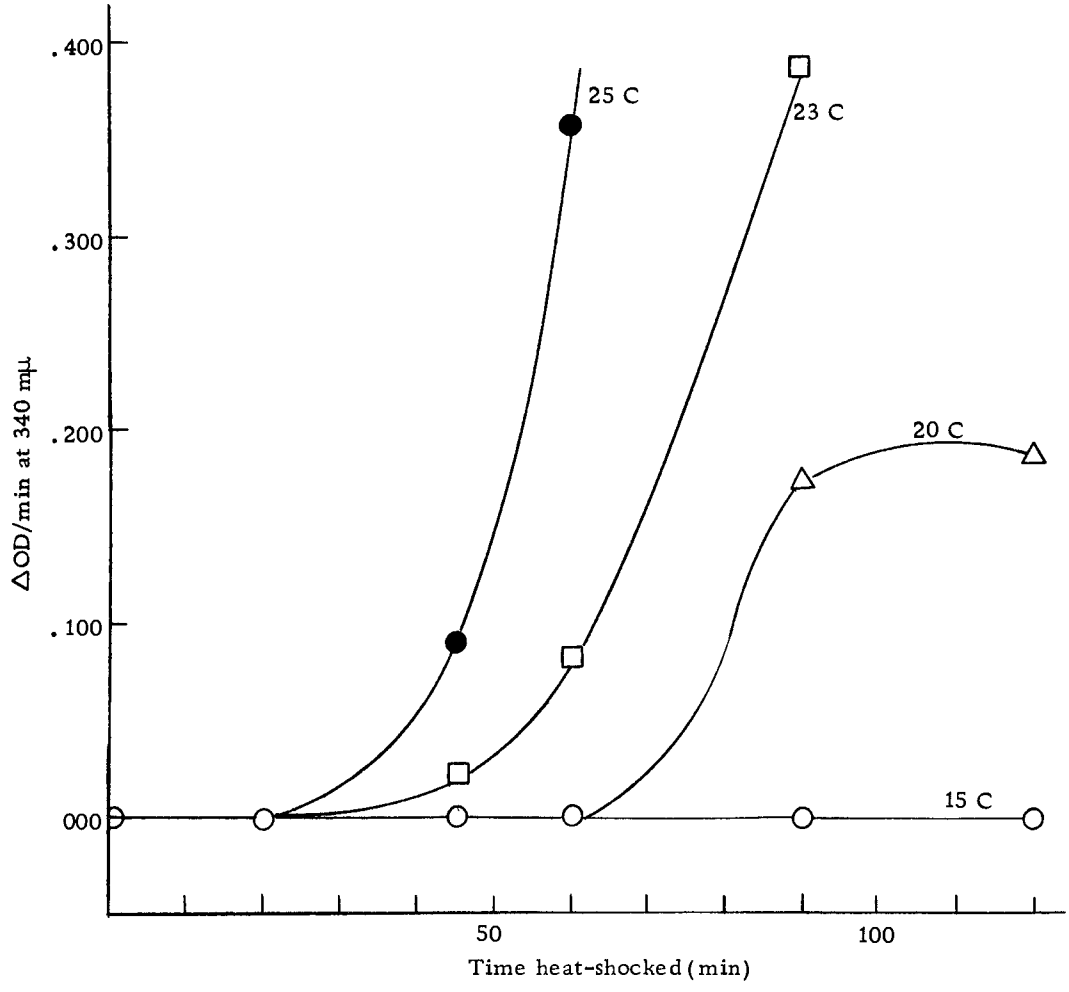

Figure 11. Leakage of malic dehydrogenase from V. marinus cells (obtained from the log phase) when heat-shocked at various temperatures in SDB medium in relation to time.

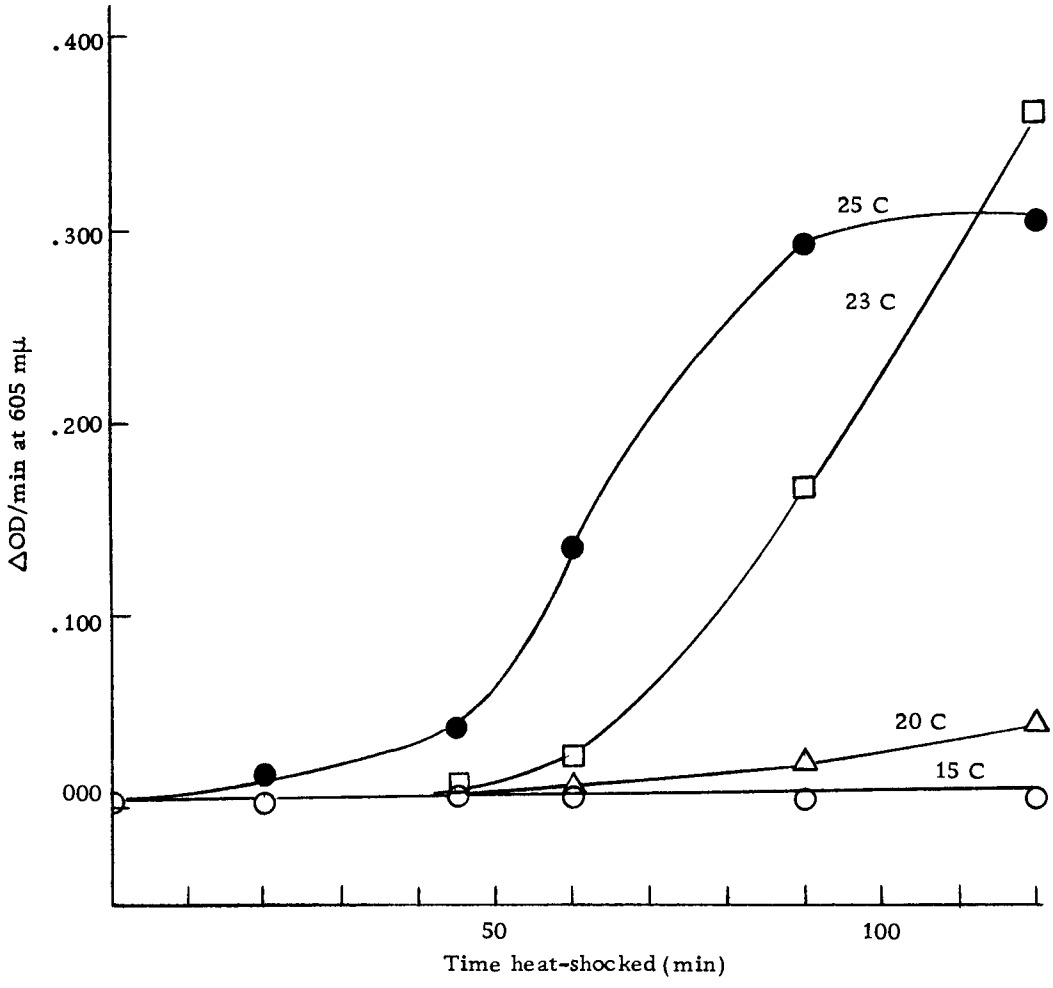

Figure 12. Leakage of glucose-6-phosphate dehydrogenase from V. marinus cells (obtained from the log phase) when heat-shocked at various temperatures in SDB medium in relation to time.

Hagen et al. (7) have shown that death occurred before lysis in another psychrophilic marine bacterium. From these data for V. marinus MP-1, it appears that leakage followed death, and lysis and leakage occurred concommitantly. Figures 5 and 13 show the relation between death, leakage, and lysis when cells were heat-shocked in SDB medium at 25 C. Only after 95 percent of the cells were rendered nonviable was leakage detected (Figure 5), with leakage and lysis occurring simultaneously (Figure 13). Phase photomicrographs of heat-shocked log phase cells are shown in Figure 14. The lighter, less dense cells (ghosts), have been lysed. Figures 15 and 16 illustrate the same trend for 20 C heat-shocked cells. Leakage was initiated when approximately 94 percent of these cells were rendered nonviable (Figure 15) with leakage and lysis occurring simultaneously (Figure 16).

At this point it may be useful to redefine leakage and lysis. Lysis is normally considered the complete dissolution of a cell. After 120 min heat-shock at 25 C the turbidity had decreased by one-half (Figure 13). Examination of these cells under the phase microscope showed almost all ghosts, which appeared as intact cells except for their less dense appearance (Figure 14). It is apparent there had not been complete dissolution of the cell wall. Lysis in V. marinus MP-1 shall be considered to be the decrease in turbidity of a suspension or the production of ghosts. Leakage will

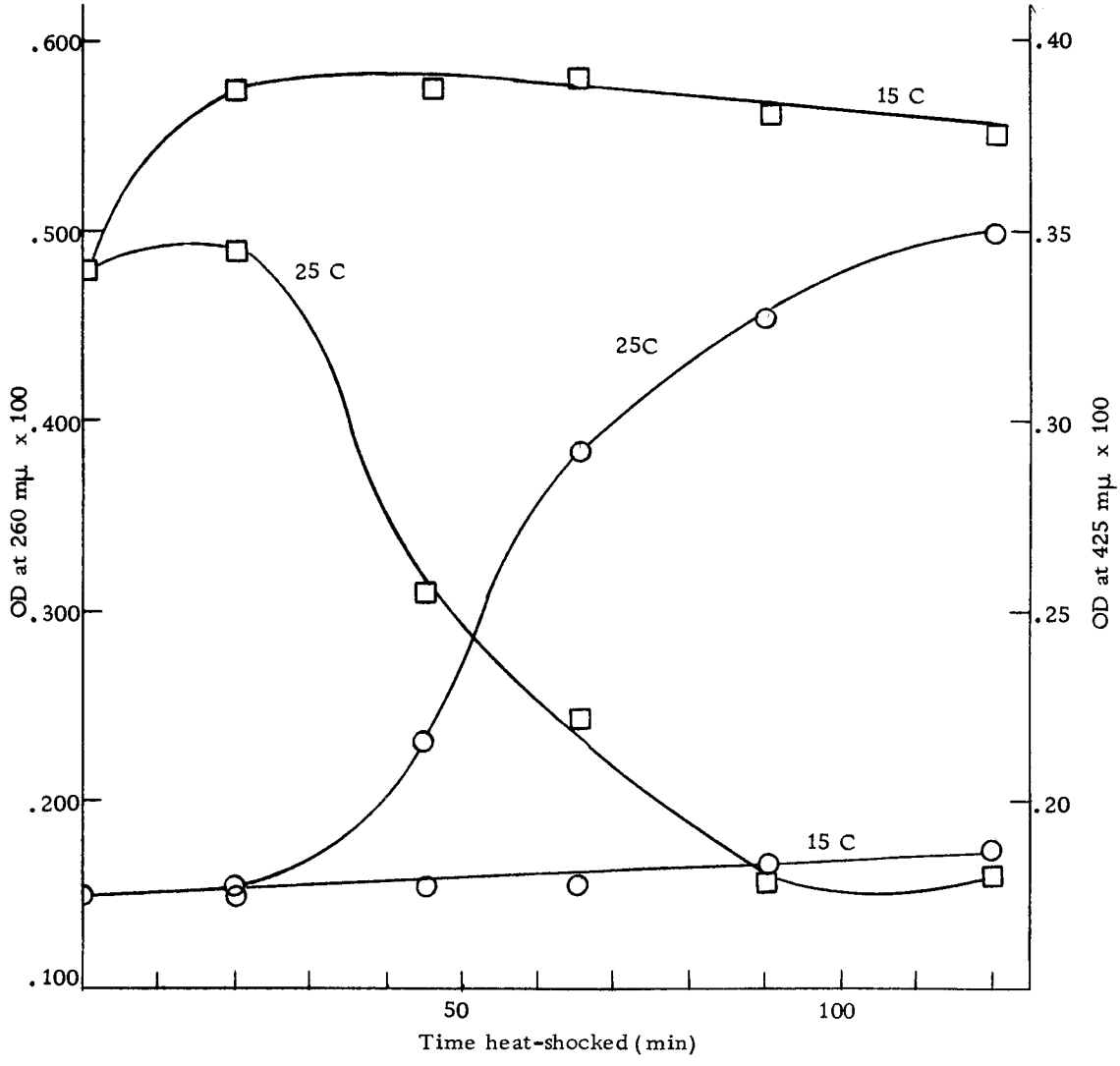

Figure 13. Leakage (○) and lysis (□) as a function of time when log phase cells were heat-shocked in SDB medium at 25 C.

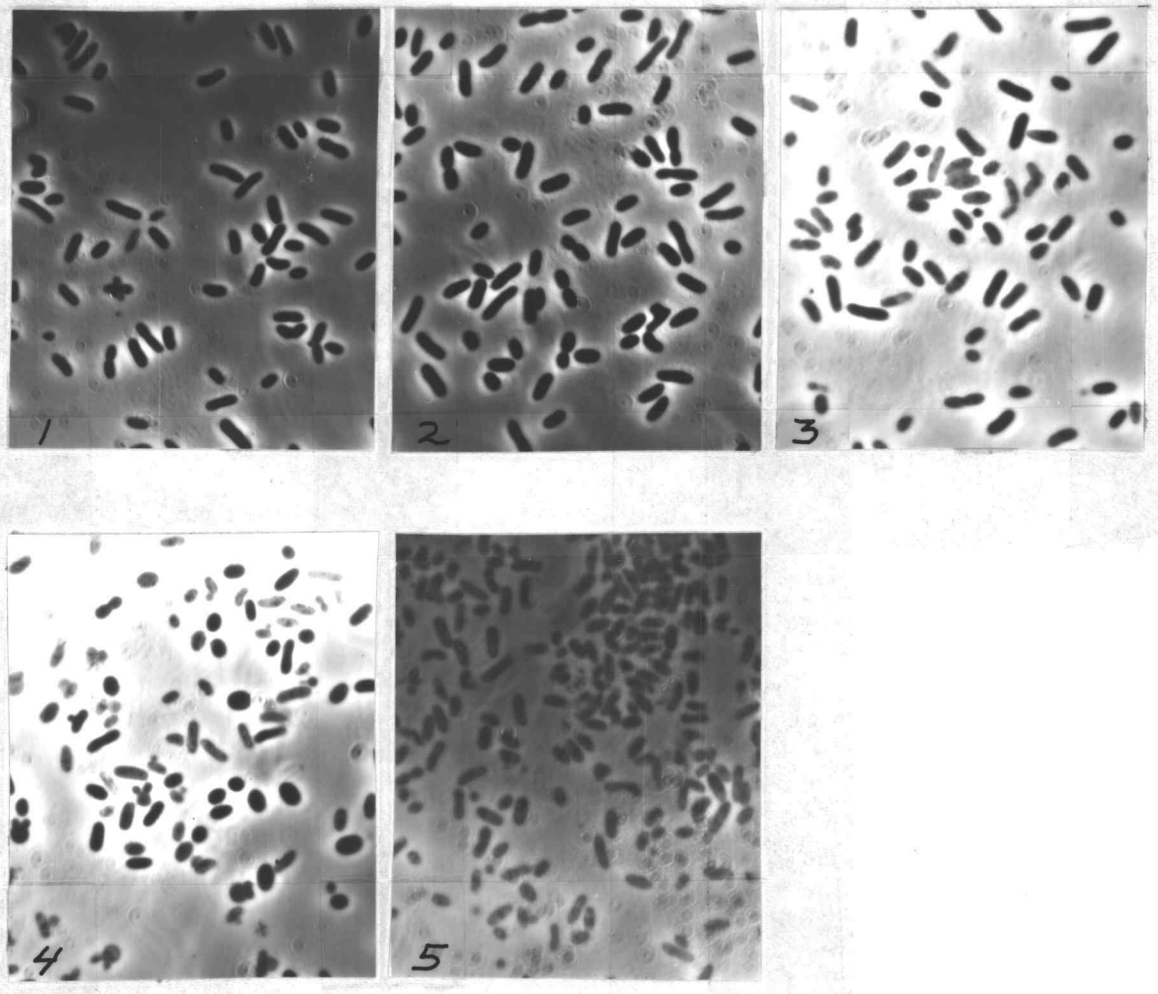

Figure 14. Phase photomicrographs of log phase V. marinus cells heat-shocked in SDB medium at 25 C for (1) 0 min; (2) 20 min; (3) 45 min; (4) 90 min; (5) 120 min.

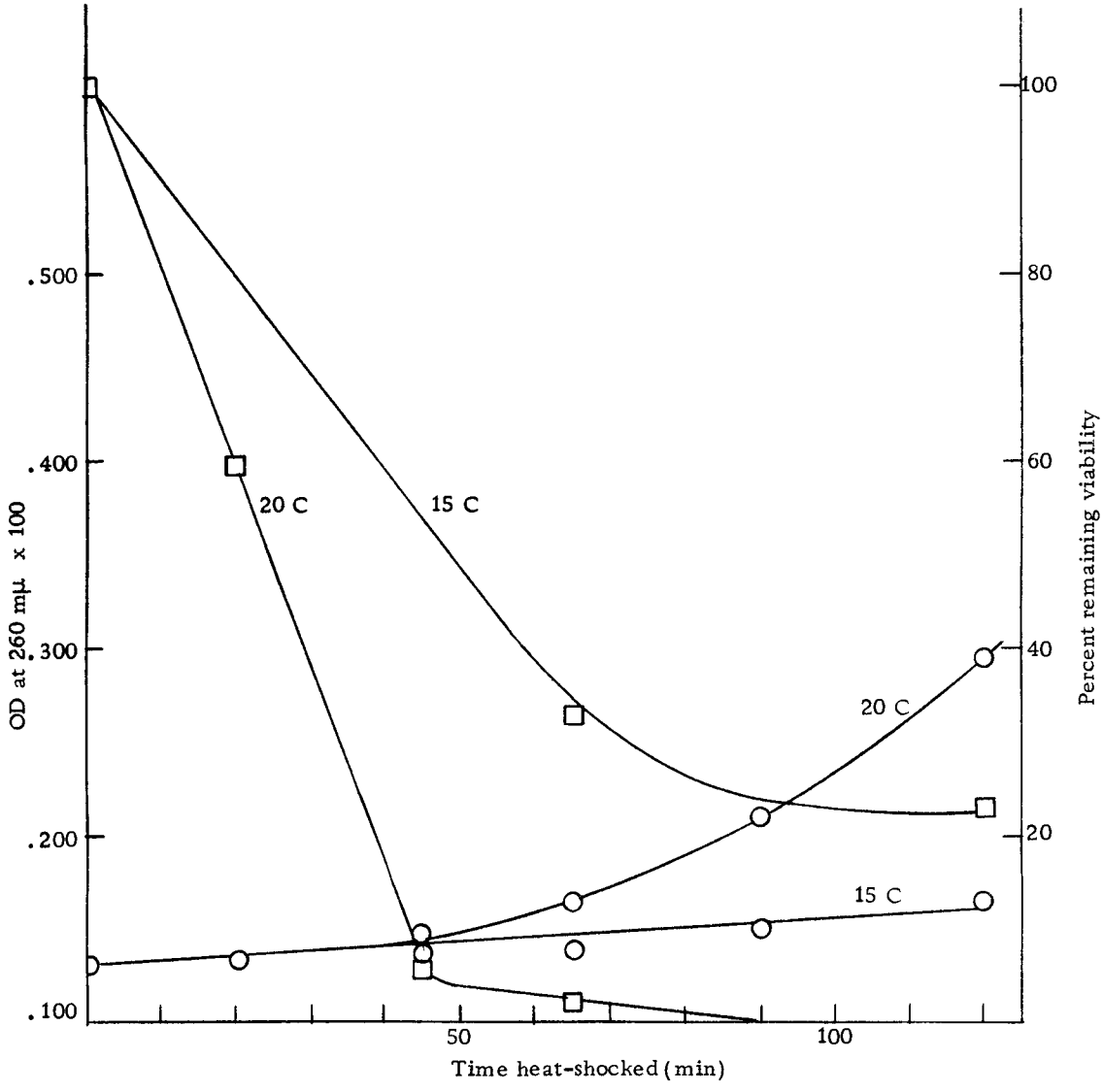

Figure 15. Viability (□) and leakage (○) as a function of time when log phase cells were heat-shocked at 20 C in SDB medium. One hundred percent viability is equivalent to 79 x 10^8 cells per ml.

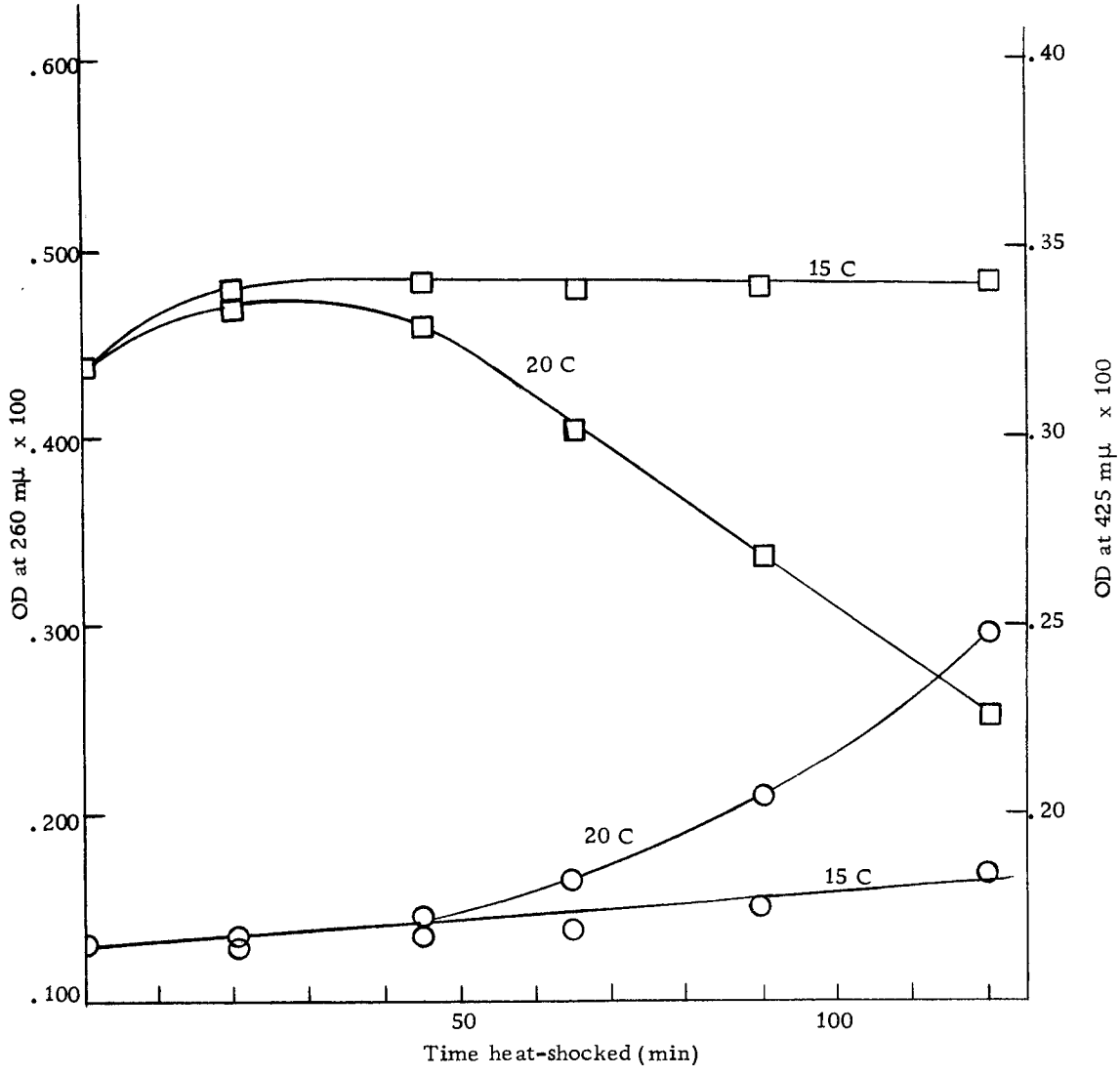

Figure 16. Leakage (O) and lysis (□) as a function of time when log phase cells were heat-shocked at 20 C in SDB medium.

be considered to be the loss of intracellular material into the menstruum with or without lysis, before or after death.

Figure 6 shows the results of heat-shocking stationary phase cells in SDB medium at 25 C. The cells were more heat resistant as discussed previously, did not leak, and were therefore more resistant to lysis. There was little measurable leakage after 120 min when more than 99.9 percent of the cells were rendered non-viable (Figure 6), with no apparent increase in the frequency of ghosts (Figure 17).

Cells grown in SOS medium increased exponentially for approximately 17 hr before approaching the stationary phase (Figure 18). When cells were heat-shocked in SOS medium at 25 C without concentration, both log phase cells and negative growth acceleration phase cells did not leak measurable 260 mμ absorbing material (Figures 19 and 20). SOS medium was used because of its low absorbance at 260 mμ. Increases in 260 mμ absorbing material could be read directly, and therefore determined without dilution of the supernatant from the heat-shocked cells. Fifty percent of the log phase cells were killed after 55 min and 99 percent after 120 min (Figure 19). Fifty percent of the negative growth acceleration phase cells were killed after 90 min and 93 percent after 120 min (Figure 20). Apparently log phase cells (Figure 19) did not leak or lyse sufficiently to increase the 260 mμ absorbing properties of the supernatant within the 120 min heat-shock period. This may be due

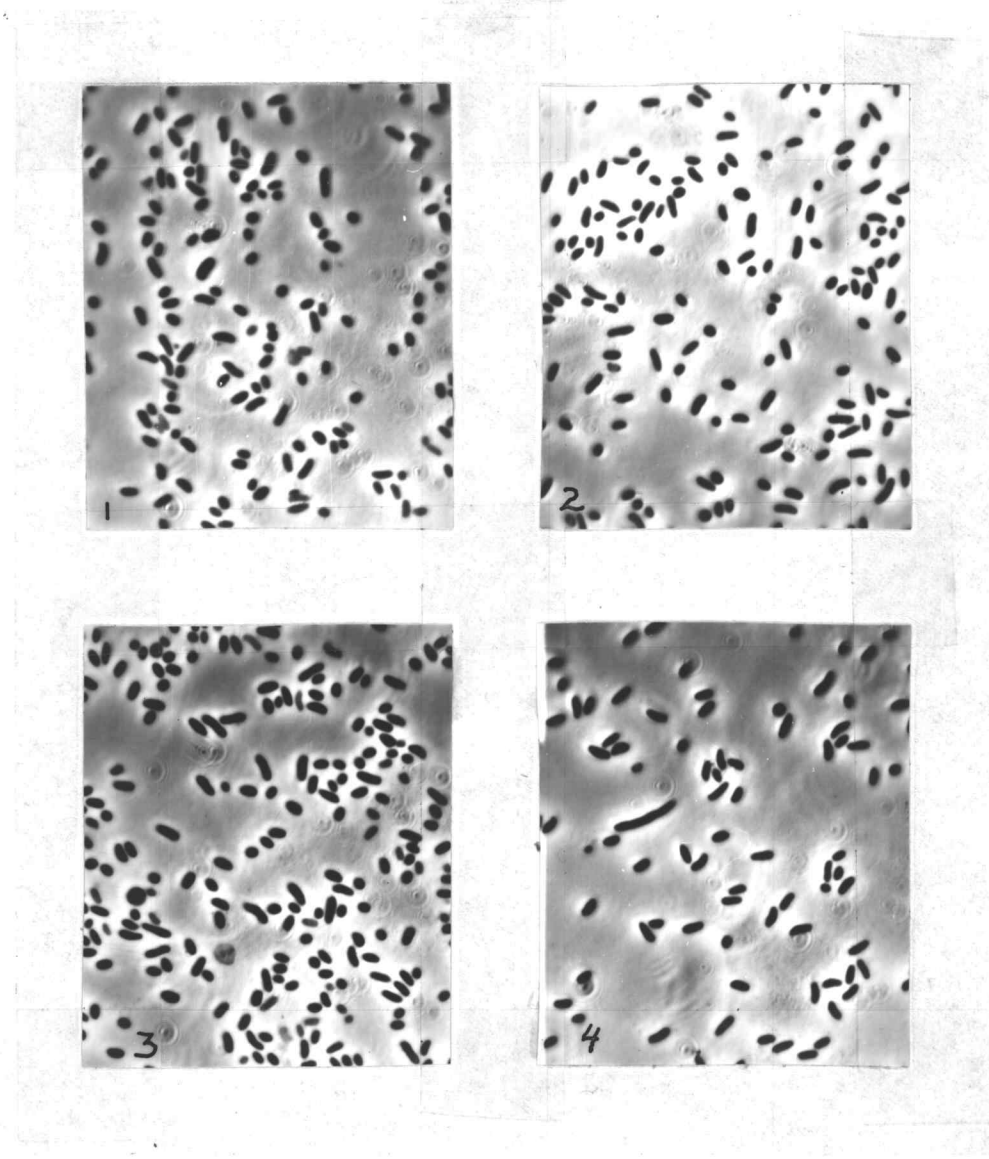

Figure 17. Phase photomicrographs of stationary phase V. marinus cells heat-shocked in SDB medium at 25 C for (1) 0 min; (2) 30 min; (3) 60 min; (4) 120 min.

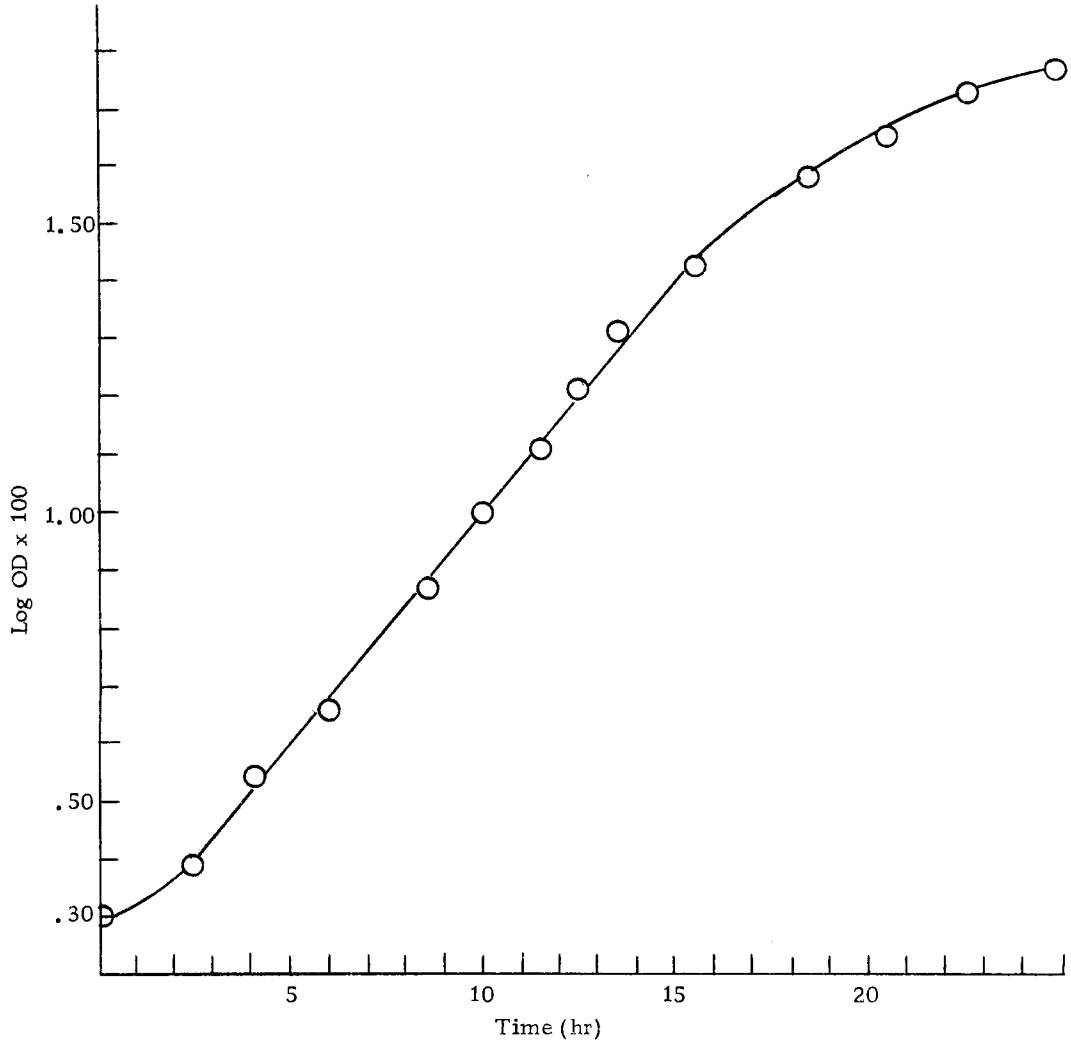

Figure 18. Growth curve of V. marinus in SOS medium.

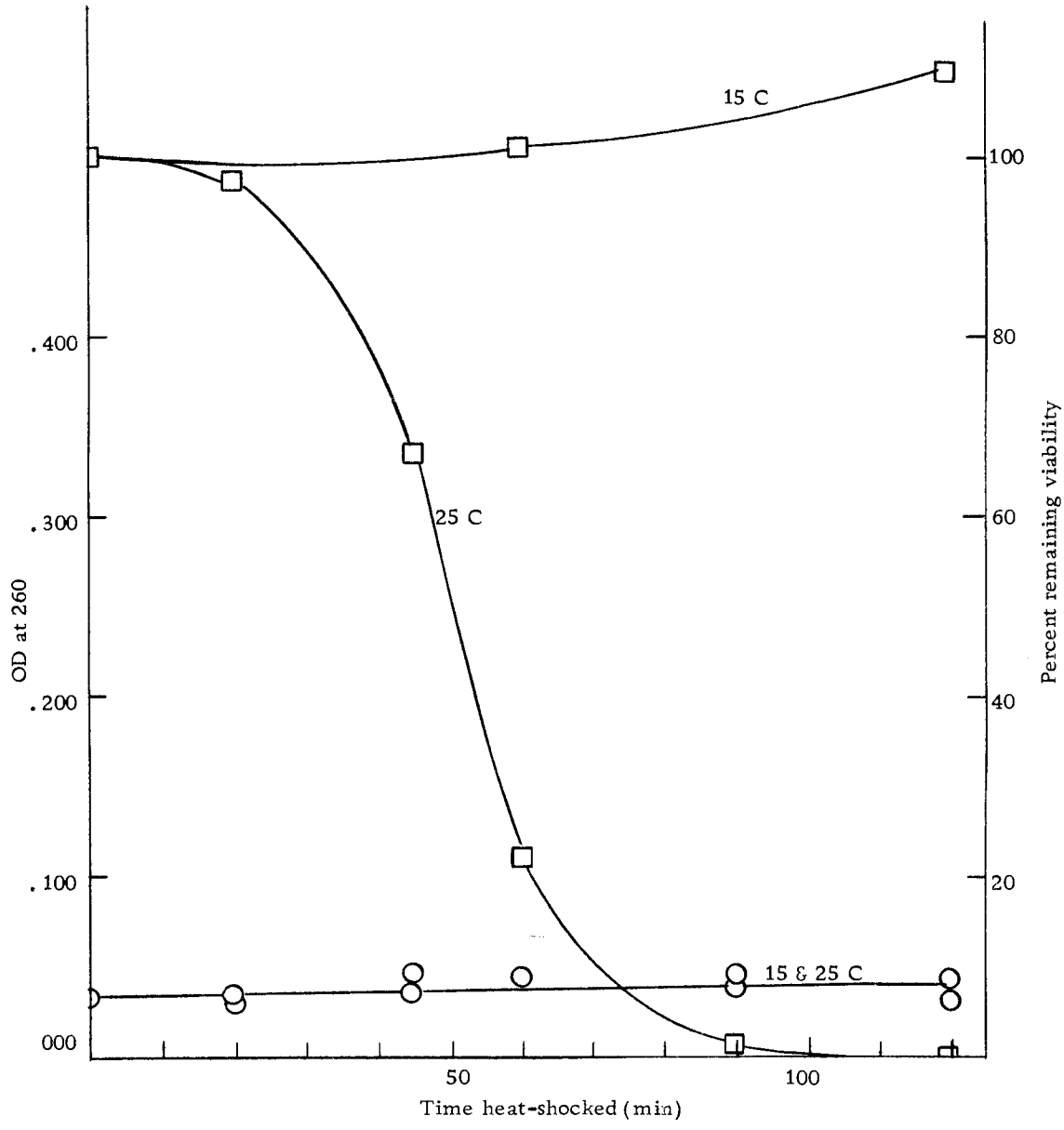

Figure 19. Viability (□) and leakage (○) as a function of time when log phase cells were heat-shocked at 25 C in SOS medium. One hundred percent viability is equivalent to 61 x 10^6 cells per ml.

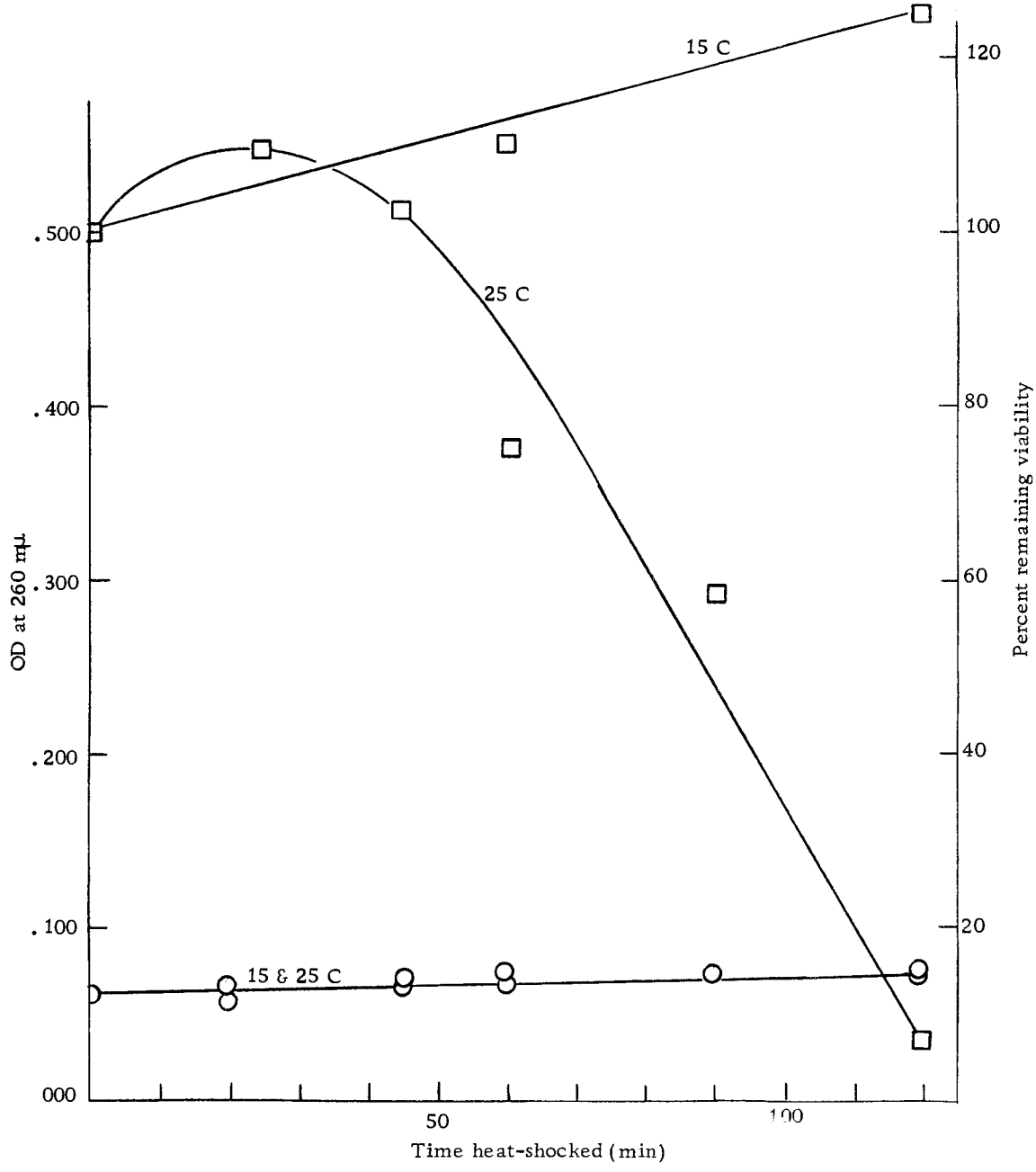

Figure 20. Viability (□) and leakage (○) as a function of time when negative growth acceleration phase cells were heat-shocked at 25 C in SOS medium. One hundred percent viability is equivalent to 36×10^7 cells per ml.

to their greater resistance to autolysis after death in these less dense suspensions. All previous leakage experiments in SDB medium utilized cells concentrated approximately 30 times. Preliminary work, not included here, indicated no increase in 260 mμ absorbing material could be demonstrated when cells from any part of the growth curve were heat-shocked for 120 min at 25 C in SDB medium; concentration of the cells was necessary.

V. marinus MP-1 was rendered nonviable by heat-shocking at 25 C. Heat-shocking was also associated with cell lysis and leakage. This investigation has demonstrated that there was a differential sensitivity of this organism to heat treatment depending solely on the age of the culture.

The data obtained from this investigation has shown that the resistance of older cultures to thermally induced death, lysis and leakage was not related to the build-up of protective substances in the medium. The factor(s) responsible for the reduced heat sensitivity must, therefore, lie within the cell itself. It is possible that the synthetic mechanisms of the cell, especially those in the extremely actively growing phase of the growth curve, are the most susceptible to heat. Since death occurs before lysis and leakage, the synthetic mechanisms of an actively growing culture may be one of the first mechanisms destroyed by heat treatment, thereby causing death of the cells.

SUMMARY

Vibrio marinus MP-1, an obligate psychrophilic marine bacterium, was killed when heat-shocked at 20 C. Cells heat-shocked at 20, 23, and 25 C released 260 mμ absorbing material (nucleic acids), orcinol reacting material (RNA), ninhydrin reacting material (amino acids), malic dehydrogenase, and glucose-6-phosphate dehydrogenase increasingly at higher temperatures. Older cultures were found to be more resistant to thermal death, lysis and leakage. No significant leakage or lysis could be detected after heat-shocking stationary phase cells in medium for 120 min when more than 99.9 percent were killed. Cells in the log phase of growth were the most sensitive to death, leakage and lysis. After 95 percent were killed at 25 C, and 94 percent at 20 C, cells began to release intracellular materials. Leakage and lysis occurred concommitantly after death. Loss of membrane permeability control before death would, therefore, not be indicated. It is suggested that the increased sensitivity to heat in young cultures may be attributed to thermolabile synthetic mechanisms involved in rapidly growing cultures.

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