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Title SOME PHYSIOLOGICAL EFFECTS OF NEAR MAXIMUM GROWTH TEMPERATURES ON AN OBLIGATELY PSYCHROPHILIC MARINE BACTERIUM

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The heat inactivation of the psychrophilic marine bacterium, Ant-300, was investigated in terms of permeability control, glucose uptake, and respiration. Extensive leakage of ^{14}C-cellular material occurred at 13 C, the maximum growth temperature of the organism. Protein and RNA were released from the cells in significant quantities. Inhibition of glucose uptake also occurred at 13 C when the cells were suspended in artificial seawater. Higher temperatures increased both the rate and extent of inhibition. The presence of nutrients in the cell menstruum afforded some protection to the uptake mechanism against heat injury. In addition, nutrients increased cell viability above the maximum growth temperature. CO_2 evolution at and slightly above the maximum growth temperature appeared to be inhibited more by a limited supply of glucose as substrate than by direct heat damage to the respiratory system. Starvation, resulting from extensive leakage of intracellular material as well as inhibited substrate uptake, was sug-

gested as a cause for the organism's inability to grow at temperatures above 13 C.

SOME PHYSIOLOGICAL EFFECTS OF NEAR MAXIMUM GROWTH TEMPERATURES ON AN OBLIGATELY PSYCHROPHILIC MARINE BACTERIUM

by

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INTRODUCTION

Bacterial growth has been noted at temperatures as high as 90 C and as low as -10 C (Brock, 1967; Ingraham and Stokes, 1959). No one organism, however, has been found to grow over this entire temperature range. As a result, organisms have been grouped into thermal classes based on their cardinal temperatures. Various investigators have dealt with the problem of what physiological factor(s) determines the upper temperature limits for survival of the bacterial cell. To date, a number of temperature sensitive cellular processes have been revealed. However, the extent to which they contribute to the actual inactivation of the organism has not yet been clearly established.

Of the various cellular components, the cell membrane has been indicated as a primary site of thermal sensitivity (Haight and Morita, 1966). More specifically, elevated temperatures have been shown to grossly disrupt the function of selective permeability across the membrane (D'Aoust and Kushner, 1971). Kenis and Morita, (1967) and Haight and Morita (1966) found that leakage of 260 nm absorbing material occurred in heat treated cells. However, the release of intracellular material could only be detected after 95 percent of the cells were killed. It was difficult, therefore, to determine whether membrane damage was a cause or an effect of cellular death.

This study is a re-investigation of the effects of moderate temperatures on psychrophilic bacteria employing the more sensitive

isotopic tracer technique. The subtle changes brought about by moderate temperatures can be better analyzed by this method thereby permitting a better interpretation of the events taking place. The heat sensitivity of permeability control, substrate uptake, and respiration in the obligately psychrophilic marine bacterium, Ant-300, are analyzed in this regard.

REVIEW OF LITERATURE

Psychrophilic bacteria have been indicated as important agents in the mineralization processes occurring in marine waters (Morita and Burton, 1970). Approximately 90 percent of the water in the ocean is less than 5 C. Despite the extremely low temperatures, these microorganisms appear to be quite well adapted to function in this environment. However, when these cold-loving bacteria are subjected to moderate temperature conditions, death quickly ensues. (ZoBell and Conn, 1940). Various investigators have attempted to determine what unique properties these organisms possess that make them so sensitive to heat. Enzyme inactivation (Evison and Rose, 1965; Langridge and Morita, 1966; Bluhm and Ordal, 1969), ribonucleic acid degradation (Allwood and Russel, 1967; Strange and Shon, 1964), membrane damage (Tomlins et al., 1972; Alsobrook et al., 1972), and inactivation of the respiratory system (Robison and Morita, 1966) have been proposed as explanations in this regard.

Evison and Rose (1965) noted that the activities of many tricarboxylic acid cycle enzymes decreased after transferring cells from optimum temperature to 1 C above the maximum for growth. Langridge and Morita (1966) demonstrated the inactivation of the enzyme malic dehydrogenase at the maximum growth temperature of 20 C in Vibrio marinus MP-1. In the mesophile, Escherichia coli, Ron and Davis (1971) showed that the decreased growth rate of the organism at elevated temperatures was due to a decrease in activity of the first enzyme of the methionine pathway. Bluhm and Ordal (1969) also noticed decreased enzyme activity in heat injured cul-

tures of Staphyloccus aureus. They suggested that, although the injury was not lethal, complete recovery did not occur, indicating some of the damage was irreversible.

Heat damage sustained by specific enzymes has been shown to affect the respiratory activity of both psychrophiles and mesophiles. Robison and Morita (1966) described a decrease in oxygen uptake with time at temperatures slightly above the maximum growth temperature in Vibrio marinus MP-1. During sublethal heat treatment of S. aureus, a mesophile, Bluhm and Ordal (1969) noted that reduced rates of substrate metabolism occurred. They suggested that the activities of several heat labile enzymes involved in substrate catabolism were responsible for a 65 percent decrease in endogenous respiration. Evison and Rose (1965), in addition to detecting a rapid decline in respiration of endogenous reserve material, noted a similar decrease when exogenous glucose and pyruvate were added at 3-5 C above the maximum growth temperature.

In many organisms, glucose metabolism appears to be a key factor in the cell's response to elevated temperatures. Iandolo and Ordal (1966) demonstrated that glucose was one of several substrates required for the reconstruction of nucleic acid pools lost across the cytoplasmic membrane of S. aureus during sublethal heat treatment. They proposed that the oxidation of glucose supplied the energy necessary for pool reconstruction. However, glucose alone could not bring about repair. Bluhm and Ordal (1969) later suggested that a decrease in the activity of certain heat sensitive enzymes involved in the conversion of glucose to a utilizable energy form inhibited the ability of the organism to repair other cellular damage

brought about by heat treatment.

There is considerable evidence indicating that membrane damage also results from exposing cells to above maximum growth temperatures (Morita and Burton, 1963). Tomlins et al., (1972) indicated that extensive phospholipid biosynthesis occurred during the recovery of heat injured Salmonella typhimurium. The synthesis of new material suggested that the original phospholipids were destroyed or released. Essentially all of the phosphorylated lipid fraction of the cell was associated with the cytoplasmic membrane.

Leakage of cellular material at elevated temperatures has provided further evidence that the membrane is a primary site of heat damage. The release of cations, amino acids, and proteins from heated cells has been observed by various investigators (Iandolo and Ordal, 1966; Allwood and Hugo, 1971; and Haight and Morita, 1966). Evison and Rose (1965) observed the leakage of UV absorbing compounds at 3-5 C above the maximum growth temperature of several marine psychrophiles. Similar leakage material was noticed by Hagen et al. (1964) during a decrease in turbidity (indicating lysis) of yet another heat treated psychrophile. Haight and Morita (1966) identified this UV absorbing material to be both polymeric and non-polymeric ribonucleic acids along with lesser amounts of deoxyribonucleic acid. They suggested that thermally induced selective leakage may represent one of the physiological bases of psychrophily.

Based on work carried out on a mesophile, Allwood and Russel (1967) suggested that cellular leakage may be due to a breakdown

of RNA within the organism, a phenomenon which has also been attributed to elevated temperatures (Sogin and Ordal, 1967; Gronlund and Campbell, 1965; Maruyama and Okamura, 1972) According to Strange and Shon (1964), RNA breakdown, which precedes bacterial death, occurs to an increasingly greater extent as the temperature of incubation increases at temperatures below the maximum growth temperature. Between 30 and 47 C the rate of RNA degradation in Aerobacter aerogenes increased linearly with temperature. Further evidence that RNA degradation occurs at elevated temperatures was presented by Tomlins and Ordal (1971) who found that protein and RNA synthesis occurred in S. typhimurium during recovery from sublethal heat injury. It was suggested that RNA degradation and leakage are similarly affected and may be related as a result of exposure to elevated temperatures.

Iandolo and Ordal (1966) proposed the following order of events with respect to thermally induced leakage. There is weakening of the cell membrane with coincident loss of soluble pools. Simultaneously, there is degradation and loss of RNA across the membrane.

The relative order in which these heat sensitive cellular processes become inactivated has received additional attention. According to Hagen et al. (1964), leakage followed lysis and lysis followed death at elevated temperatures in an obligate psychrophile. It was suggested that death was neither due to lysis nor to cell

wall breakdown or breakdown of the cell membrane. In V. marinus MP-1, Kenis and Morita (1968) determined that leakage followed death, and lysis and leakage occurred concomitantly at a temperature 5 C above the maximum growth temperature in nutrient medium. Allwood and Russel (1967), on the other hand, proposed that leakage occurred before death and lysis in S. aureus. They based their reasoning on the fact that during heat treatment, leakage was observed before a decrease in viability occurred.

The apparent differences noted above in the order of events effecting inactivation of the various organisms may result from subtle differences by which the cells were cultured during analysis. Such factors as nutrient availability, cell concentration, and the degree and time of heat shock have been shown to affect the degree and order in which these cellular events respond to temperature (Strange and Shon, 1964).

Various investigators have demonstrated the effects of nutrient availability on the survival of microorganisms at elevated temperatures (Tomlins and Ordal, 1971; Iandolo and Ordal, 1966). Robison and Morita (1966) and Haight and Morita (1966) found that the presence of organic nutrient material extended the viability of V. marinus MP-1 to temperatures slightly above the maximum growth temperature. The death rate of the mesophile A. aerogenes was also dependent upon the composition of the heating medium as well as several other parameters (Strange and Shon, 1964)

More specifically, there is evidence indicating that nutrient availability has a direct effect on particular physiological processes

in the cell at higher temperatures (P. R. Kenis, M.S. Thesis, 1967). Kenis demonstrated that leakage and an increase in UV absorbing material occurred to a greater extent in rich nutrient medium than in a limiting nutrient medium. Strange and Shon (1964) observed that leakage was not as extensive in starved cells as in unstarved cells. Such observations indicated that in spite of the fact that nutrient availability enhanced the survival of cells exposed to elevated temperatures, the leakage of intracellular material occurred to a lesser extent when cells were deprived of a nutrient source.

A comprehensive study of "nutrient protection" on Bacillus psychrophilus revealed that a diverse set of compounds were effective to various degrees in retarding lysis in heat treated cells (Mattingly and Best, 1972). D-serine, sucrose, and polyethylene glycol were effective in this regard. The presence of sucrose has also been shown to reduce leakage at elevated temperatures in S. aureus (Allwood and Russel, 1967). Although interactions with the osmotic mechanism are implied, the precise nature of protection afforded by these compounds has yet to be explained.

MATERIALS AND METHODS

Organism

The organism employed in this study is a recent isolate recovered during Cruise #46 of the U.S.N.S. Eltanin. The isolate, referred to in this thesis as Ant-300, was recovered in cold Antarctic waters from a depth of 300 meters. Ant-300, tentatively identified as a Vibrio species (Baross, J., personal communication), was chosen for this study on the basis of its psychrophilic properties.

Growth Temperature Range

The temperature range of growth for Ant-300 was determined in a polythermostat. The growth medium consisted of glucose, 2.5 g; sodium nitrate, 0.5 g; Bacto-yeast extract (Difco), 0.2 g; Tris buffer, 1.2 g; Rila Marine Mix (Rila Products, Teaneck, New Jersey), 40.0 g; distilled water, 1 liter. The pH was adjusted to 7.8.

Early log phase cells (OD = 0.25 at 600 nm) diluted 1:1000 were transferred into ten ml of glucose medium equilibrated over a gradient of temperatures. Cell growth at these temperatures was determined as an increase in OD at 600 nm using a Bausch and Lomb Spectronic 20 colorimeter.

Viability of Heat Shocked Cells

Viability of Ant-300 during heat treatment was measured by plate counts. The term "viability" in this thesis is defined as the ability to produce a colony when transferred to Sheril D.

Burton (SDB) agar medium at 5 C. This medium consisted of glucose, 0.5 g; succinic acid, 0.2 g; sodium chloride, 15.0 g; Rila Marine Mix, 5.0 g; polypeptone (BBL), 5.0 g; Bacto-yeast extract, 3.0 g; ferrous sulfate, 0.005 g; Difco agar, 15.0 g; distilled water, 1 liter. The pH was adjusted to 7.8. Plates were incubated for 3-5 days before counting.

Heat treatment, in this text, refers to incubating the cells near or above the maximum growth temperature. Cells exposed to heat treatment were suspended in two different menstruums. One was a complex medium described above for Growth Temperature Range studies with the deletion of glucose. The second was an artificial seawater solution composed of 4 percent Rila Marine Mix in distilled water with the pH adjusted to 7.8.

Early log phase cells diluted 1:100 were transferred into flasks containing the desired suspending menstruum. The flasks were incubated at near maximum (13 C) and above maximum (15 and 18 C) growth temperatures in New Brunswick psychrotherm incubator shakers (23 mm amplitude at 80 strokes/min.). At ten hour intervals portions were removed, diluted, and plated on SDB agar medium. Plates were incubated at 5 C for 3-5 days before counting.

Pulse Labeling Studies

Early log phase cells (OD = 0.25) grown in an unlabeled glucose medium were diluted 1:100 into a basal salts medium composed of Rila Marine Mix, 40.0 g; sodium nitrate, 0.5 g; Bacto-yeast extract,

0.2 g; distilled water, 1 liter. The pH was adjusted to 7.8. Glucose-UL-^{14}C was added to a final concentration of 6.0 μg/ml with an activity of 0.2 μCi/ml. The culture was incubated in a psychrotherm shaker at 5 C for 22 hours (approximately 2 generations) after which the labeled glucose not taken up was removed by washing the cells. Cold artificial seawater (sterilized by filtration) was employed in all washing procedures. After washing, the cells, in this case, were resuspended to original volume with wash solution. A final concentration of approximately 2 x 10^6 bacteria/ml was obtained. The cells were thoroughly resuspended on a shaker at 5 C for one hour.

Release of Labeled Material from Heat Treated Cells

Five-ml portions of the resuspended cells were transferred to sterile 16-mm test tubes and sealed with rubber serum caps pierced with a plastic rod and cup assembly (Kontes Glass Co., Vineland, N.J., K-882320) containing 30 x 50 mm strips of Whatman no. 1 filter paper. The test tubes were divided among three psychrotherms equilibrated to 13, 15, and 18 C. A 5 C control was included to correct for normal leakage and respiration. At ten hour intervals duplicate tubes were removed from each incubator and the quantities of label in the CO_2, menstruum, and cell fractions were determined.

Recovery of radioactivity as $^{14}CO_2$

The samples were acidified to a pH of 2.0 (0.1 ml of 1 N sulfuric

acid/5 ml of cell suspension) after which 0.2 ml of β-phenyethylamine was injected into the cup assembly containing the filter paper. Any $^{14}CO_2$ present in the tube was adsorbed onto the filter paper at 5 C. After one hour of adsorption the filter paper was removed and placed into a scintillation vial containing toluene fluor (0.03% triphenyl-dioxazol and 0.5% 2,5-diphenyloxazole in toluene).

Recovery of radioactivity in the cells and menstruum

Separation of the cells from the menstruum was accomplished by centrifugation at 9150 x g for ten minutes at 0 C. One ml of the supernatant was placed into a scintillation vial containing a two parts toluene fluor and one part Triton X-100. This mixture was employed in all instances in which the radioactivity was in an aqueous suspension. The remaining supernatant and cell pellet from each centrifuge tube were rinsed with artificial seawater and filtered through a 0.45μ HA membrane filter (Millipore Corp.). The cells adsorbed on the filters were dried and placed in scintillation vials containing toluene fluor. All vials were counted in a Mark I Nuclear Chicago scintillation counter. A quench curve was employed to correct for quenching in each sample. Sufficient counts were obtained to give a probable counting error of 0.48 percent.

Calculations involving respiration and leakage

The amount of radioactivity incorporated into the pulse labeled cells was calculated as follows:

Percent respired = (DPM as $^{14}CO_2$/total DPM incorporated by cells) x 100.

$$\text{Percent leakage} = \frac{\text{DPM/ml of supernatant x volume of supernatant}}{\text{total DPM incorporated by the cells}} \times 100.$$

Analysis of Leakage Material

Larger volumes of cellular leakage material were obtained through the following alterations to the previous procedure. Following resuspension of the washed cells in artificial seawater, the culture was distributed equally among two Erlenmeyer flasks. One flask was incubated at 5 C as a control while the other was incubated at 18 C. After four hours of aeration the contents of each flask were centrifuged at 9150 x g for ten minutes at 0 C. The supernatant fluid was decanted and immediately frozen.

Dialysis of leakage material

Twenty ml of thawed supernatant was distributed into dialysis tubing, sealed, and placed in a beaker containing chilled artificial seawater for 36 hours at 5 C. The contents of the tubing were adjusted to the original volume by adding artificial seawater. One ml of the dialyzed supernatant was placed into a scintillation vial containing fluor and the radioactivity was counted. The remaining dialyzed supernatant was frozen and stored for later analysis.

Protein and DNA determination of leakage material

Protein determination was carried out by the method of Kennel (1967). The procedure employed 25 mm glass pre-filters (Millipore Corp.) to separate out the labeled acid precipitable protein fraction. By adding additional protein to the sample the efficiency of recovering the small amounts of radioactive protein as leakage material was increased. This was accomplished by adding 50 μg/ml of Bovine Serum Albumin, fraction 5, (Sigma Chemical Co.) to the sample before filtration. The filters holding the labeled protein fraction were air dried and placed into scintillation vials containing toluene fluor and the radioactivity counted. The fraction of leakage material present as ^{14}C-labeled protein was determined as follows:

Percent of the leakage material present as protein =

$$\frac{\text{DPM/ml of supernatant as protein}}{\text{DPM/ml of total leakage material}} \times 100.$$

The protein + DNA fraction retained on the filters was also measured (Kennel, 1967). The filters were dried and the radioactivity was counted as described in the protein determination. The fraction of leakage material present as labeled protein + DNA was calculated in the same manner as was the protein fraction.

RNA determination of leakage material from cells

Yeast RNA, type XI, (Sigma Chemical Co.) was added to each sample at a concentration of 50 μg/ml before the RNA was determined. The method of Kennel (1967) was used. The RNA fraction was ob-

tained by alkaline hydrolysis of the supernatant fluid. Glass pre-filters were used to separate the soluble RNA nucleotides from the insoluble DNA and protein material. One ml of the final filtrate was transferred to a scintillation vial containing the proper fluor and the radioactivity was counted. The fraction of leakage material present as ^{14}C-labeled RNA was determined as follows:

Percent of the leakage material composed of RNA =

$$\frac{\text{DPM/ml of supernatant as RNA}}{\text{DPM/ml of total leakage material}} \times 100.$$

Amino acid determination of leakage material from cells

The occurence of amino acids as cellular leakage material was determined by the procedure of Webb and Wood (1966). Desalting the supernatant was accomplished by passing a one liter sample through an ion exchange resin (Bio-Rad Chelex-100, converted to the Cu^{++} form). The amino acids present were retained by the resin while the salts and other material were eluted. A smaller column containing 2 ml of the NH_4^+ form of the resin was then attached to the larger column and the amino acids were eluted with NH_4OH. The eluant was evaporated to dryness with a Rinco flash evaporator at 65 C. The residue containing the free amino acids was suspended in 0.2 N citrate buffer (pH = 2.2) containing an internal standard of 0.25 M norleucine. The solution was analyzed through the courtesy of R. Becker on a Spinco Model 120B Automatic amino acid analyzer.

Uptake and Respiration Studies

Uptake of labeled substrate was measured in heat treated cells (5, 13, and 15 C) over a 60 hour period. Again, two types of suspending menstruum were employed as described in the cell viability study; one was composed of artificial seawater while the other was a complete medium. Cells were cultured as previously described in unlabeled glucose medium, washed, and diluted 1:100 into medium containing no glucose. Cells employed for uptake in artificial seawater were starved for 24 hours at 5 C prior to heat treatment. In the case where complete medium was used, cells were aerated for one hour following the washing procedure to insure a homogenous cell suspension.

Following resuspension or starvation, the culture was distributed among various flasks and incubated in psychrotherms at 5, 13, and 15 C. At ten hour intervals, duplicate 5-ml portions were removed and placed into tubes containing Glucose-UL-^{14}C at a final concentration of 0.9 μg/ml with an activity of 0.02 μCi/ml. The tubes were sealed as described previously in Release of Labeled Material From Heat Treated Cells and incubated for two hours before acidification to pH = 2.0. Radioactive CO_2 was collected as described previously in the leakage study.

The ^{14}C-label associated with the cells was collected on a 0.45 μ HA membrane filter and dried before counting the radioactivity. Each set of duplicate tubes was accompanied by a control tube to which the acid was added immediately following the addition of the cells and labeled substrate. Uptake of ^{14}C-glucose in complete

medium was measured in disintegrations per minute (DPM). The rate of uptake was measured in DPM of ^{14}C-glucose taken up per hour per viable cell (DPM/hr/viable cell). DPM in this case represents a quantity of glucose. The viable cells present at the onset of each 2-hour uptake period were determined by plate counts.

RESULTS

Growth Profile at Different Temperatures

The maximum growth temperature occurred between 13 and 14 C and the optimum growth temperature occurred near 7 C (Fig. 1). Although the lowest growth temperature was not determined, a high cell yield was noted at 1 C.

Greater cell yields were obtained with prolonged incubation at low temperatures (Table 1). Other Antarctic isolates that were tested displayed similar results.

Viability of Heat Treated Cells

Growth at 13 C was limited to a one order of magnitude increase over a 60 hour period (Fig. 2). In comparison, the cell number increased two orders of magnitude over the same period at 5 C. Cells heated to a temperature slightly above 13 C (ie, 15 C) experienced neither an increase nor a decrease in viability over a 60 hour period. Cells heat shocked at 18 C displayed a decrease in viability of approximately two orders of magnitude.

In artificial seawater cell viability appeared more sensitive to elevated temperatures. Figure 3 illustrates that after 20 hours at 15 C, viability decreased with time. Previously, when the cells were maintained at that temperature in a complete medium 100 percent viability occurred for at least 60 hours. No loss in viability appeared to have occurred at 13 C in spite of the absence of nutrients.

The results show that the presence of nutrients raised the

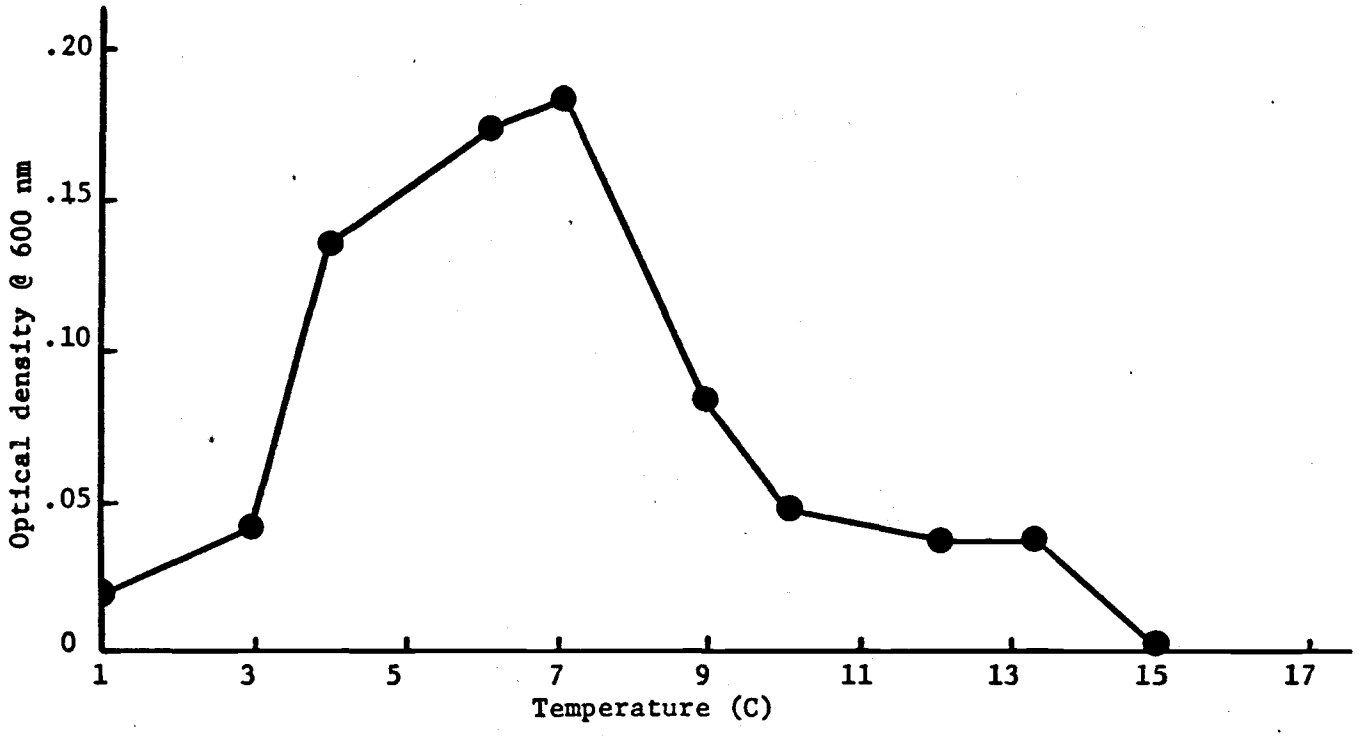

Figure 1. Growth profile at different temperatures of the Antarctic isolate, Ant-300. Cells were incubated in Glucose medium as described in Methods and the OD determined after 57 hours.

TABLE 1. CELL YIELDS OF ANT-300 AT DIFFERENT TEMPERATURES

	Optical density	
Temperature (C)	57 hours	143 hours
1.0	0.02	0.42
3.0	0.04	0.40
4.0	0.13	0.38
6.2	0.17	0.28
7.0	0.18	0.26
8.9	0.08	0.24
10.1	0.05	0.22
12.0	0.04	0.18
13.2	0.04	0.13
15.0	0.01	0.03
16.1	0	0

Cells were grown as a static culture in 0.05 percent glucose medium and the growth measured as an increase in optical density at 600 nm.

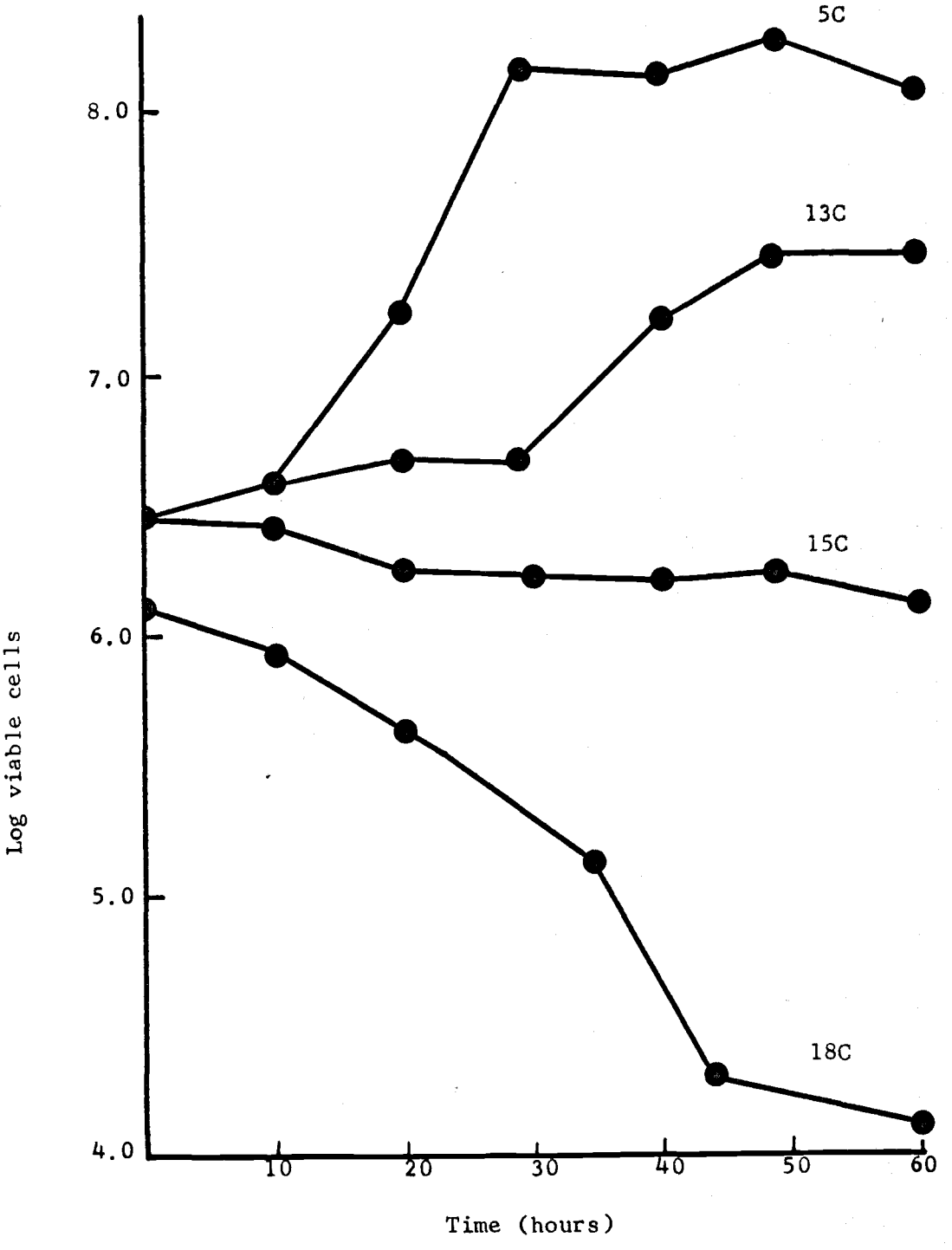

Figure 2. Time-temperature relationship in viability and growth of cells suspended in glucose medium.

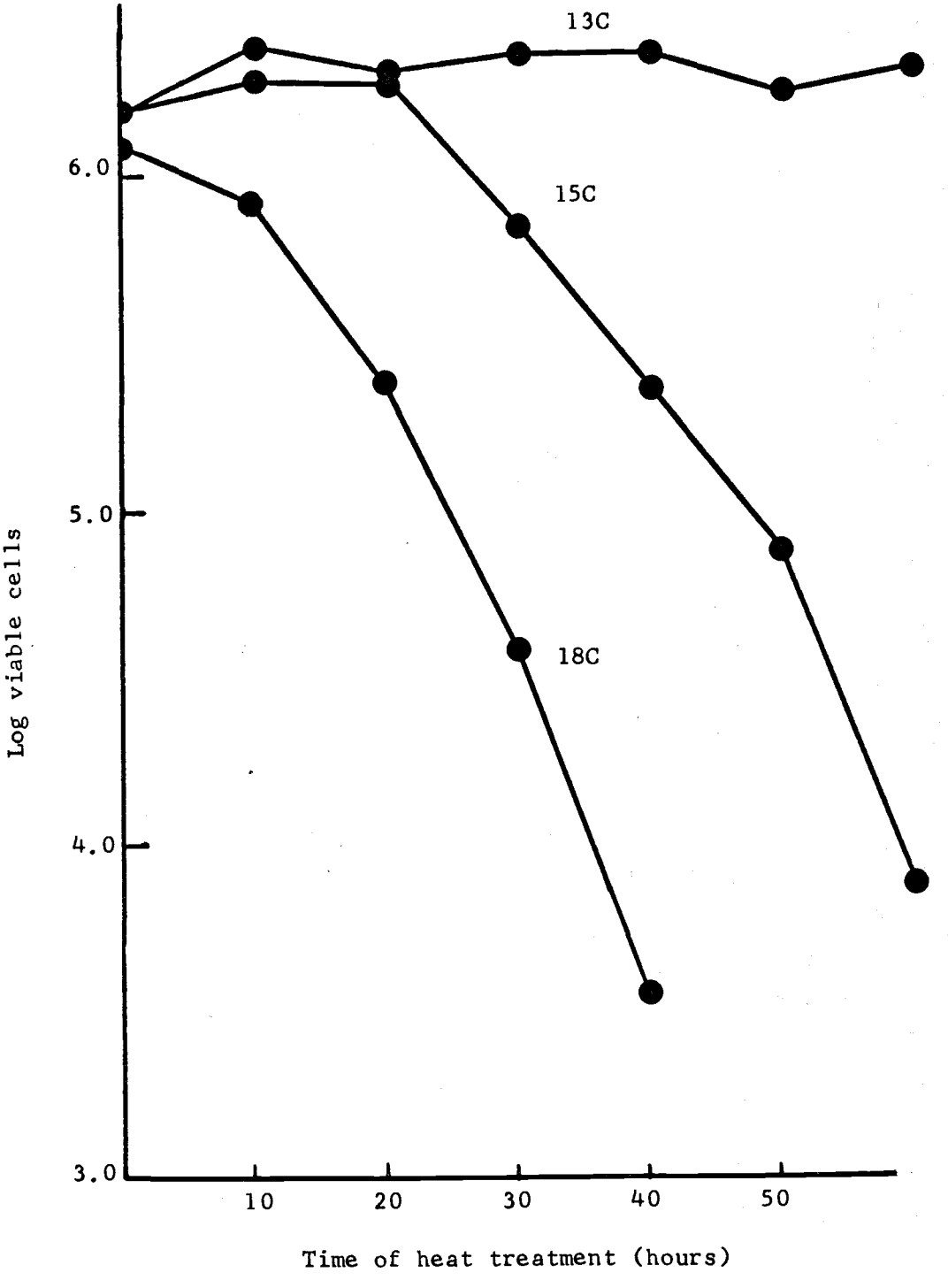

Figure 3. Time-temperature relationship on viability of cells suspended in artificial seawater.

upper limits of thermal inactivation from 13 to 15 C. Where the cells were suspended in artificial seawater, 100 percent viability was maintained at temperatures no higher than 13 C. However, if glucose and growth factors were added to the salt solution, 100 percent viability was obtained at 15 C.

Leakage from Heat Treated Cells

Figure 4 illustrates the differences in the amount of material released from the cells in artificial seawater at 13, 15, and 18 C over a 60 hour period. A control was carried out at 5 C (near optimum growth temperature) to help account for normal leakage of material from the cells.

Leakage, as evidenced by the loss of ^{14}C-cellular material, was most extensive within the first ten hours of incubation. At 18 C, 31 percent of the radioactivity was released from the cells while at 13 and 15 C, 16 percent was released. The 5 C control cells released 7 percent of the label over the same time interval. Leakage during the remaining 50 hours of heat treatment at 13, 15, and 18 C was considerably reduced.

A plateau was observed after 20 hours at 5 and 13 C after which no further release of material from the cells occurred. At 15 and 18 C, leakage continued for at least 60 hours. Therefore, after 20 hours of incubation, leakage was negligible in cells incubated at or below the maximum growth temperature. However, at temperatures above the maximum growth temperature, leakage continued over the entire period of investigation.

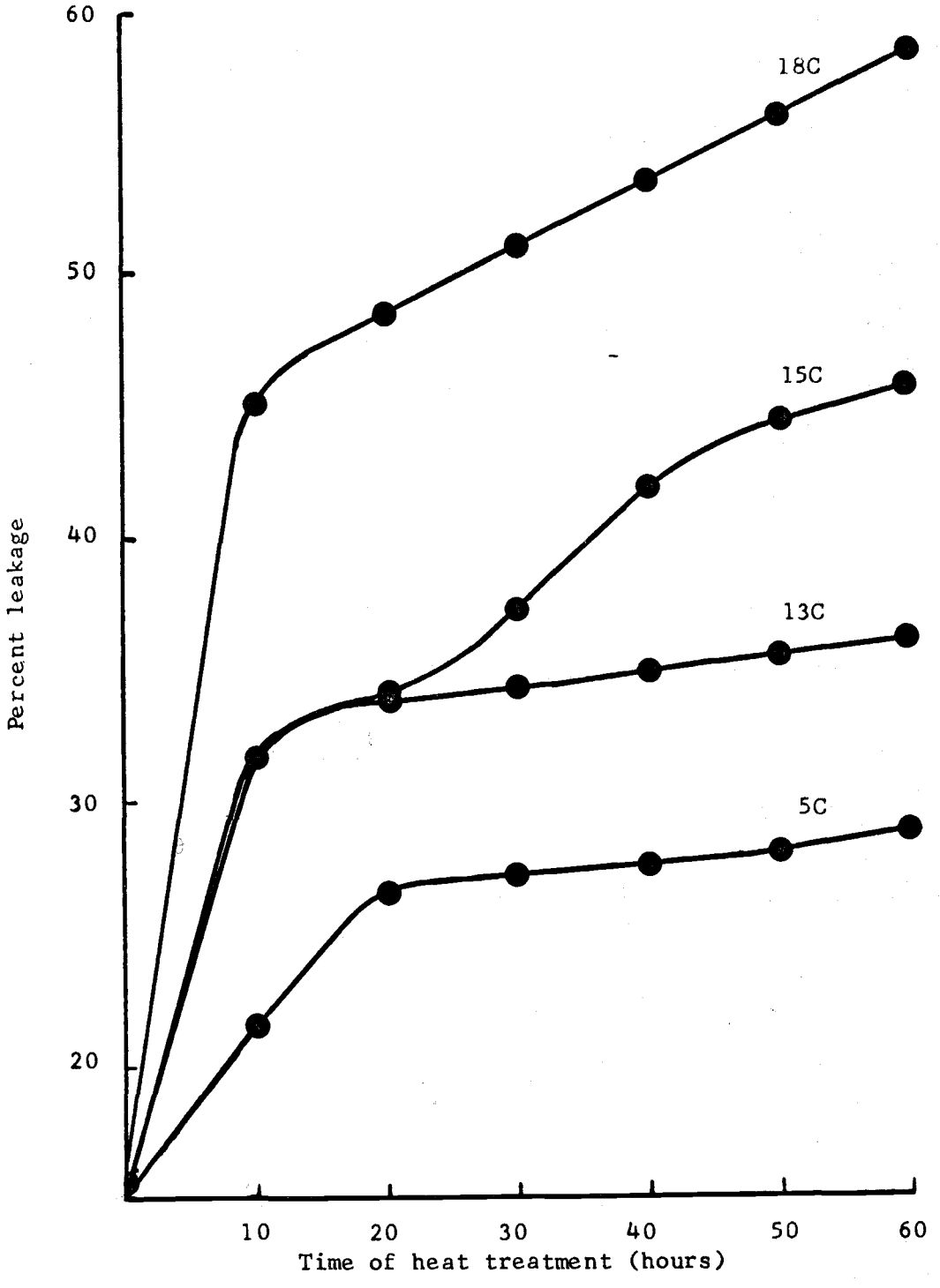

Figure 4. Release of ^{14}C-label from the cells into the suspending menstruum. Cells were subjected to various temperatures for varying periods of time.

Analysis of Leakage Material

The menstruum of cells incubated for four hours at 18 C in artificial seawater was used as a source of leakage material. The results in Fig. 5 illustrate that after four hours significant quantities of material were released from the cells even though the rate of respiration remained nearly constant and 100 percent viability was maintained. The menstruum from cells held at 5 C was employed as a control to distinguish between normal and heat induced leakage components.

Results of dialyzing the cell-free menstruum illustrate the basic nature of the cellular components leaking across the membrane of heat shocked cells. Approximately three times more leakage of labeled material occurred at 18 C than at 5 C (Table 2). However, there was only two times more non-dialyzable labeled leakage material in the 18 C menstruum than in the control. Of the total labeled material released, 20 percent was non-dialyzable at 18 C and 35 percent at 5 C. Thus, there was a qualitative as well as a quantitative difference in the material released from the cells at the two temperatures.

Several large molecular weight cellular fractions appearing in the undialyzed menstruum of normal and heat treated cells are presented in Table 3. Relative quantities of each component were compared on the basis of ^{14}C-label present in each fraction.

In the protein fraction, approximately twice as much ^{14}C-label was recovered in the menstruum of cells held at 18 C than in that of the 5 C control cells. However, the fraction of the total

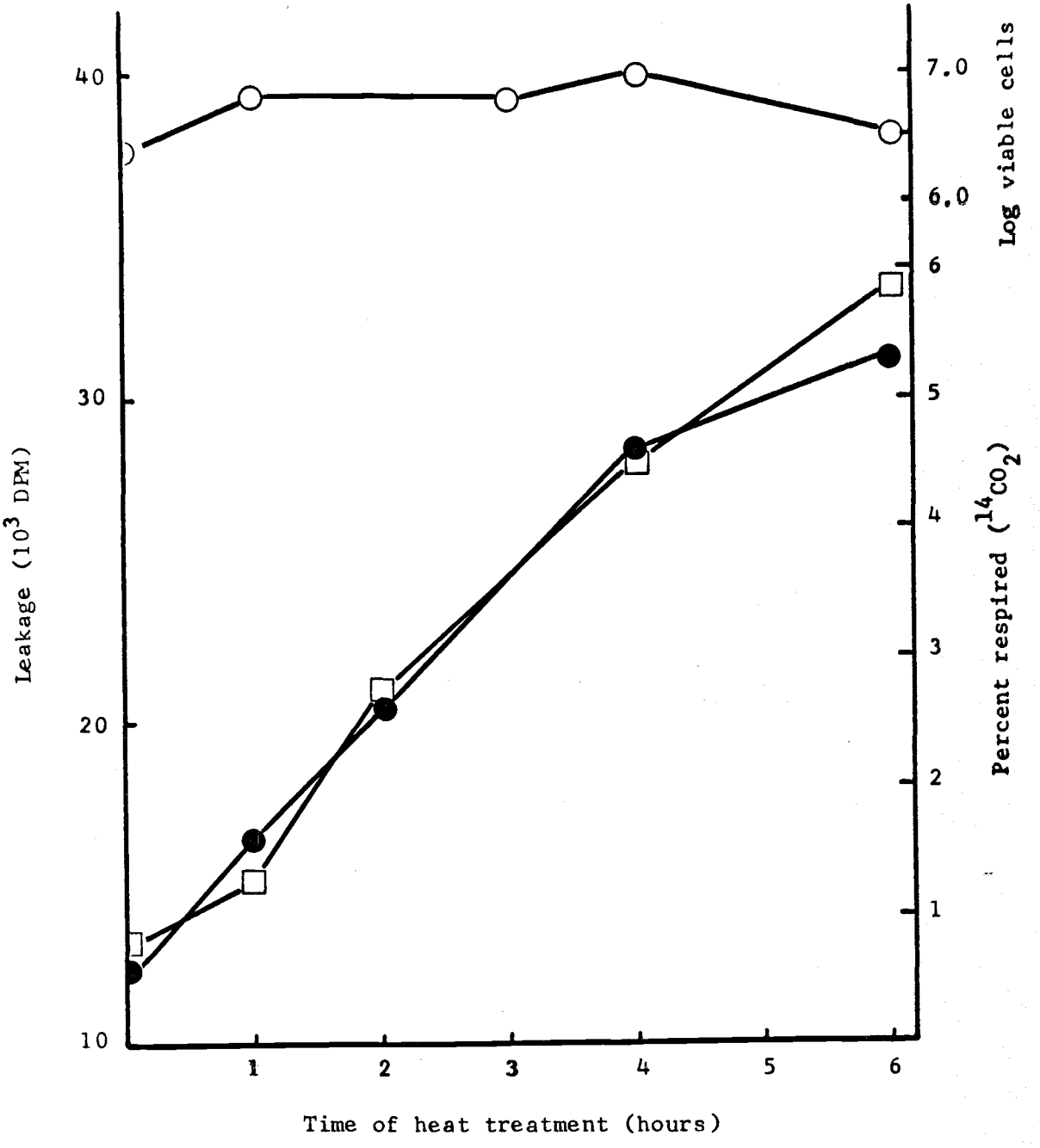

Figure 5. Leakage (□), respiration (●), and viability (○) of cells heat treated at 18 C in artificial seawater.

TABLE 2. THERMALLY INDUCED LEAKAGE MATERIAL RELEASED FROM RADIOACTIVELY LABELED ANT-300 CELLS.

Menstruum	DPM/ml before dialysis	DPM/ml of non-dialyzable fraction	Percent non-dialyzable
5 C (control)	1817	643	35
18 C heat shocked cells	5754	1158	20

The cell menstuums were dialyzed for 36 hours at 5 C. The fraction of non-dialyzable leakage material in the menstruum is shown as a percent of the total leakage material.

TABLE 3. RECOVERY OF THE VARIOUS FRACTIONS OF ^{14}C-LABELED LEAKAGE MATERIAL FROM CELLS INCUBATED AT 5 AND 18 C FOR A PERIOD OF FOUR HOURS.

Fraction	Sample	
	5 C (control)	18 C cells
Total		
Activity	2264	5161
Percent of total	100	100
Protein		
Activity	397	848
Percent of total	17	16
Protein + DNA		
Activity	409	886
Percent of total	18	17
RNA		
Activity	218	744
Percent of total	10	14

The activity was determined as DPM/ml of suspending menstruum. The activity in each fraction was also determined as a percentage of the total ^{14}C-leakage material.

leakage material present as protein was similar at both temperatures. Values of 17 and 16 percent for the 5 and 18 C menstruums, respectively were recovered.

DNA occurred as a negligible component of the total leakage material. There was less than a one percent increase of label recovered from the DNA + protein fraction as compared to that recovered from the protein fraction alone. The virtual absence of DNA in the leakage pool was also demonstrated by the diphenylamine assay (Burton, 1956).

The RNA fraction of the total leakage material is also presented in Table 3. Approximately three times more ^{14}C-leakage material appeared as RNA at 18 C than at 5 C. In addition, the RNA released at the higher temperature accounted for a larger fraction of the total leakage pool than did the RNA released in the control. An approximate value of 14 percent at 18 C as opposed to 10 percent at 5 C was noted. The less sensitive orcinol reaction, used previously by Kenis and Morita (1968) could not detect the presence of RNA in samples of leakage material identical to those used above.

An amino acid determination of the menstruum from both 5 and 18 C incubated cells indicated that very low concentrations of these compounds were present. Determination of individual amino acids in the menstruum was attempted initially by one dimensional paper chromatography as described by Clark (1964). No detectable levels of any amino acid were recovered, however. Likewise, condensation of one liter of leakage material to a volume of 3 ml did not provide enough

of any one amino acid for detection on the amino acid analyzer. Webb and Wood (1966) determined that the average recovery efficiency of the free amino acids using their technique was 40 percent. Therefore much of the leakage material present as free amino acids may have been lost in preparation.

Effects of Heat Treatment on Glucose Uptake

The effects of moderate temperatures on glucose uptake was studied in Ant-300. Uptake of ^{14}C-glucose in complete medium at 5, 13, and 15 C is presented in Fig. 6. The results indicate that the uptake system was damaged at 15 C since the level of uptake was below that observed at 13 C. Despite the damage, the rate at 15 C was always above the rate observed in the 5 C control cells. The rate of glucose transport at 13 C was at least twice that observed at 5 C at all times and at the 30 hour sampling period, the rates differed by one order of magnitude.

The reversibility of heat damage sustained by the glucose uptake mechanism was studied by shifting the heat treated cells from 13 and 15 C to 5 C immediately after adding the labeled glucose. Figure 7 illustrates that very little reversal of damage, imposed on the uptake mechanism at 15 C, occurred when the cells were returned to near optimum growth temperature.

The rate of glucose uptake at 5 C in cells heated previously at 13 C decreased to approximately 50 percent of that measured directly at 13 C. After 30 hours, the uptake rate of 13 C incubated

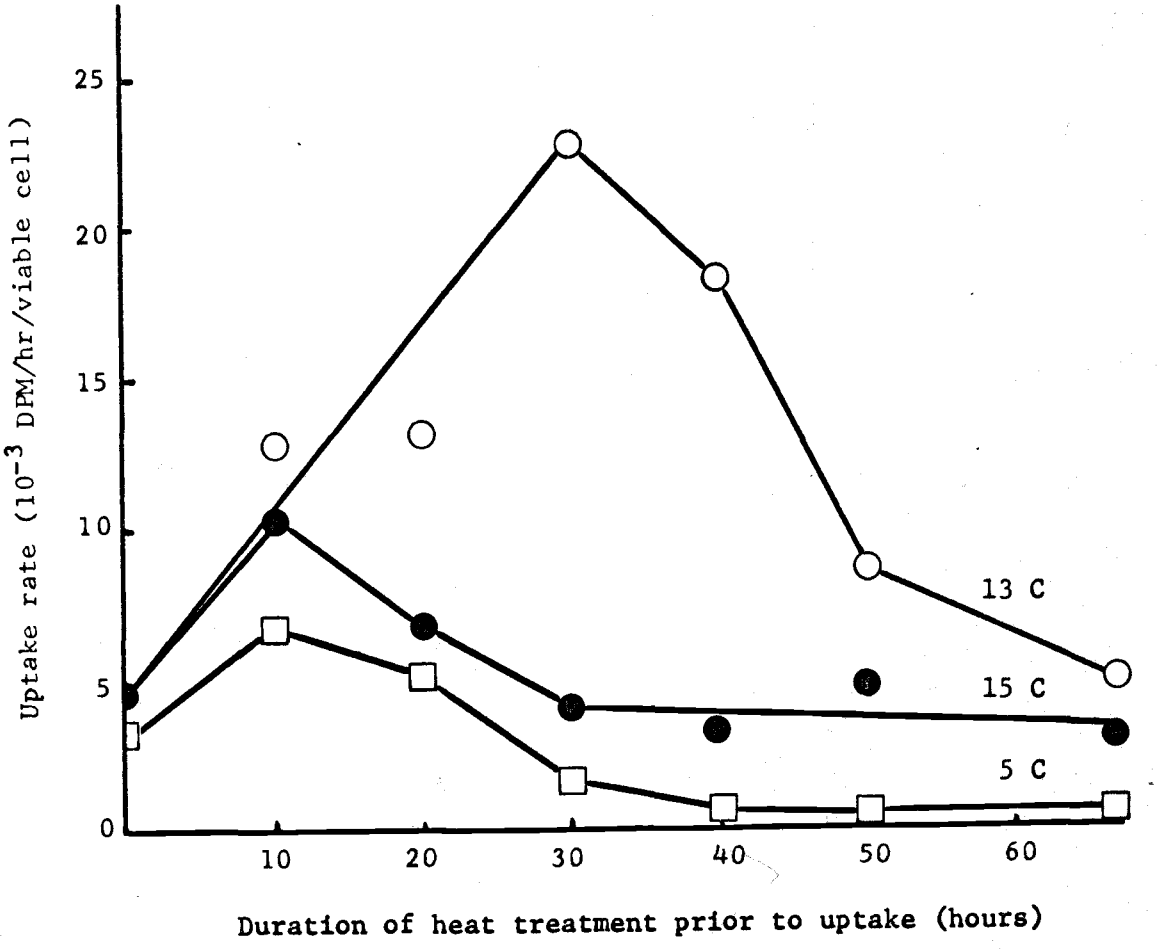

Figure 6. Uptake of ^{14}C-glucose at 5 C (□), 13 C (○), and 15 C (●) in complete medium.

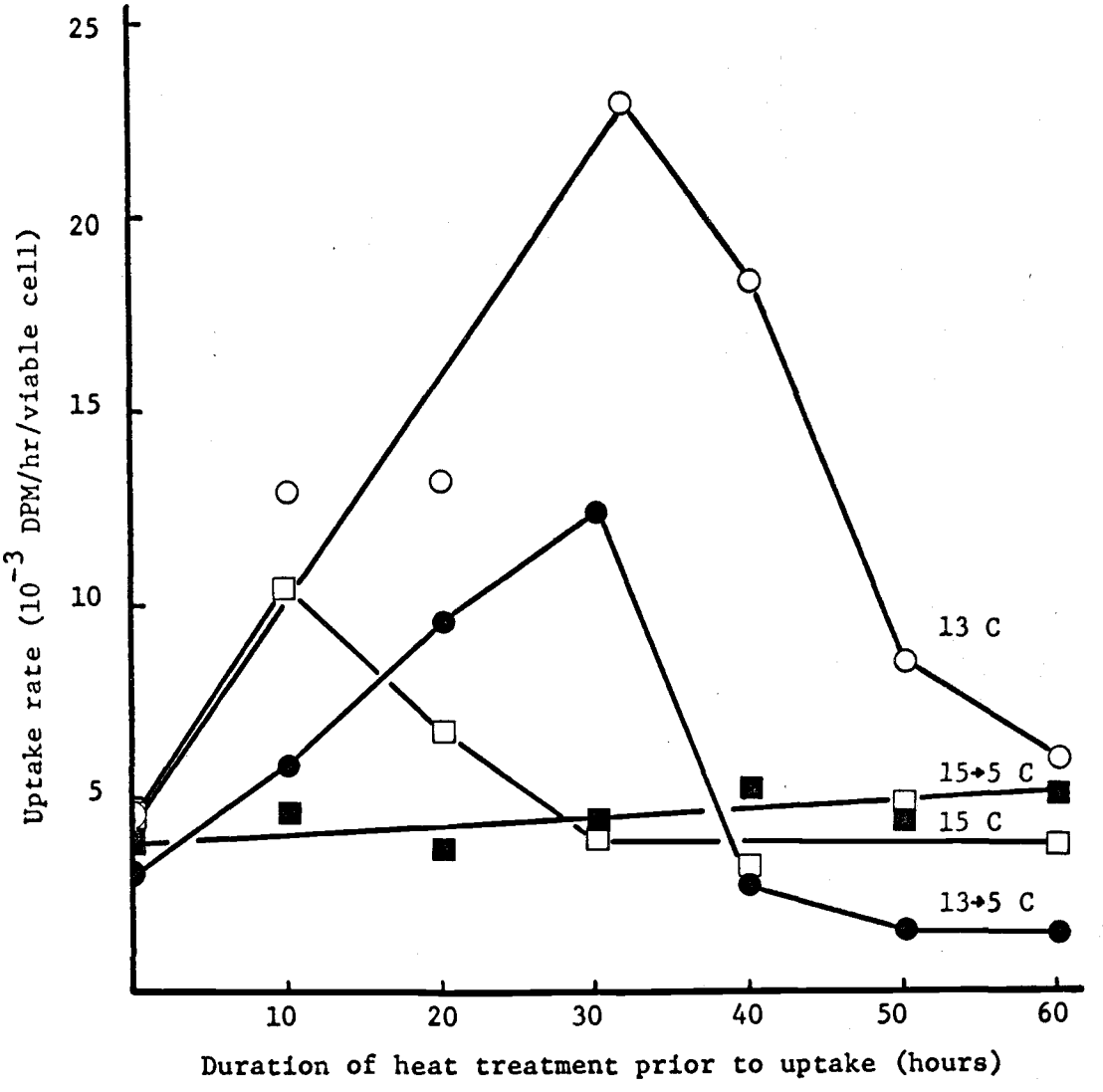

Figure 7. Uptake of ^{14}C-glucose by cells heat treated in complete medium. Uptake was determined at 13 C (○) and 15 C (□) as well as at 5 C following heat treatment at 13 C (●) and 15 C (■).

cells was less than that of 15 C incubated cells when uptake was measured at 5 C.

An apparent relationship exists between the uptake rate and the phases of cell growth at 5 and 13 C as shown in Fig. 8 and 9, respectively. At both temperatures an increase of glucose uptake began during the lag phase and maximum uptake occurred at the onset of the log phase. As the cells passed through log growth uptake dropped to a lower rate. Minimum uptake occurred in the stationary phase. The absence of growth prevented a comparison between glucose uptake and growth phase at temperatures above 13 C.

The pronounced effects of growth on substrate uptake made it difficult to examine the influence of heat on glucose transport. In an attempt to circumvent the problem, the cells were suspended in artificial seawater during heat treatment. In addition to eliminating growth, the absence of a carbon source in the incubation menstruum forced the cells to depend upon the glucose transport system as the sole means of replenishing their carbon and energy requirements when the labeled substrate was added.

Glucose uptake in cells suspended in artificial seawater at 5, 13, 15, and 18 C is presented in Fig. 10. These data indicate that in artificial seawater, temperatures as low as 13 C induced damage to the membrane resulting in decreased transport rates of glucose; higher temperatures brought about a more rapid and extensive rate reduction than did lower temperatures.

The inhibition of glucose uptake in artificial seawater at elevated temperatures was not reversed when the cells were trans-

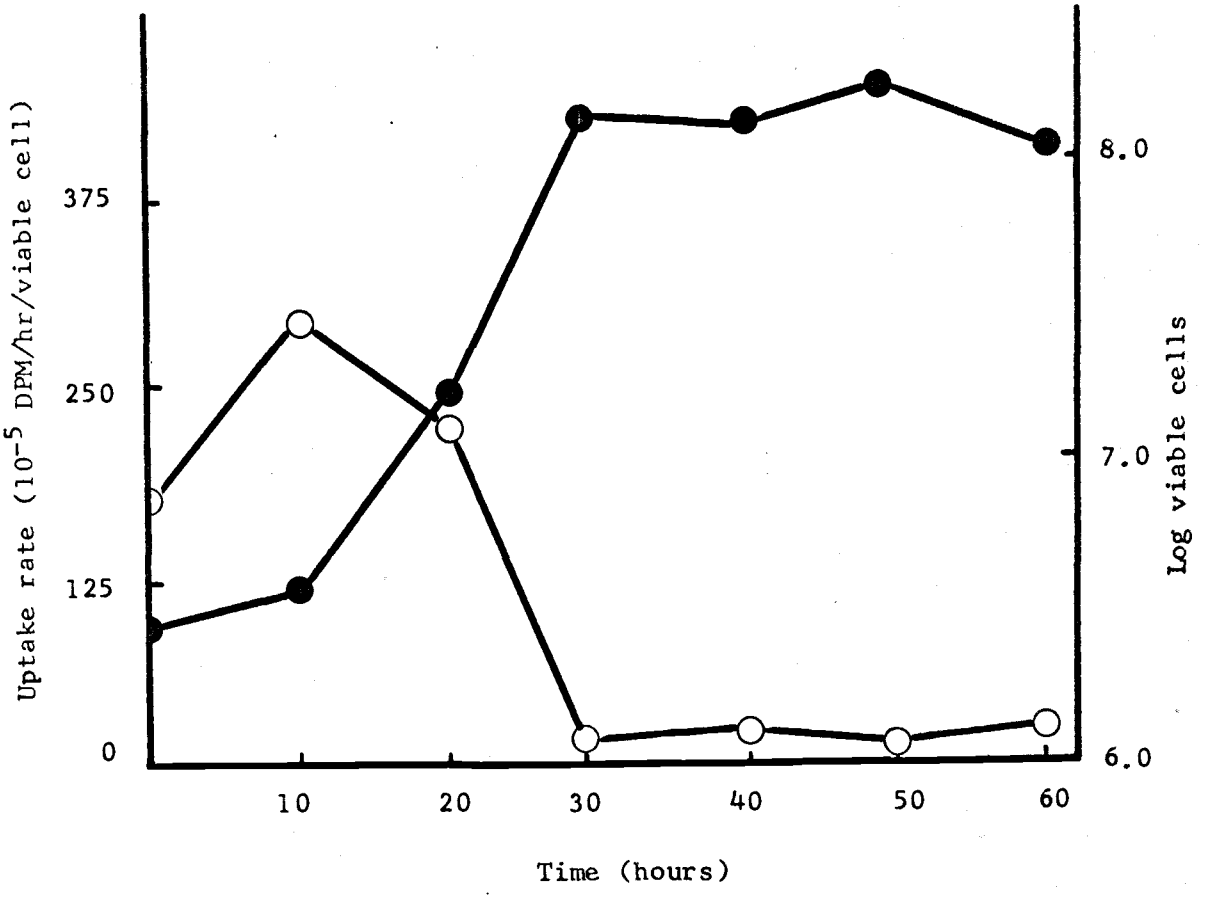

Figure 8. Relationship between uptake of ^{14}C-glucose (○) and growth phase (●) for cells incubated at 5 C.

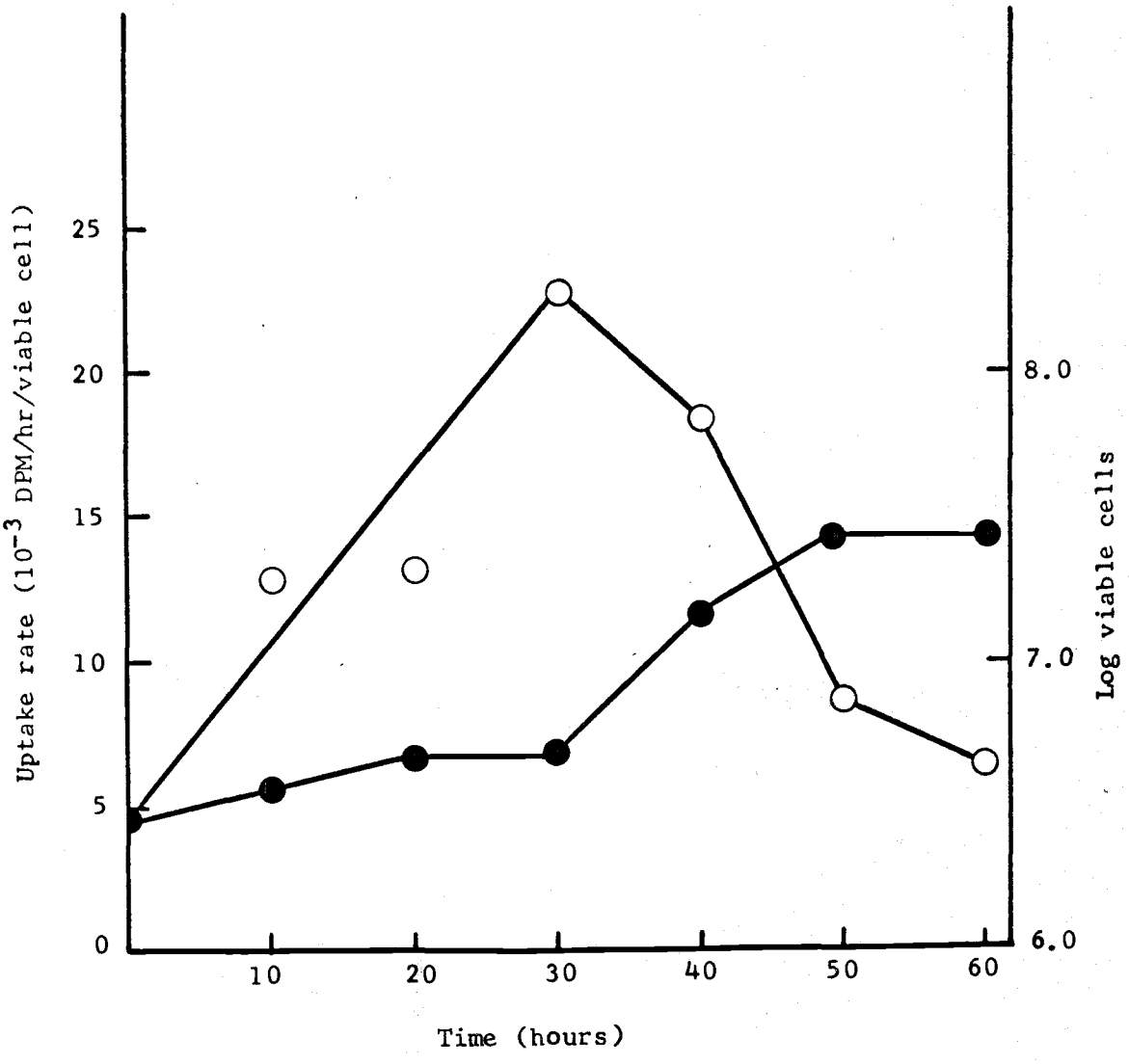

Figure 9. Relationship between uptake of ^{14}C-glucose (○) and growth phase (●) for cells incubated at 13 C.

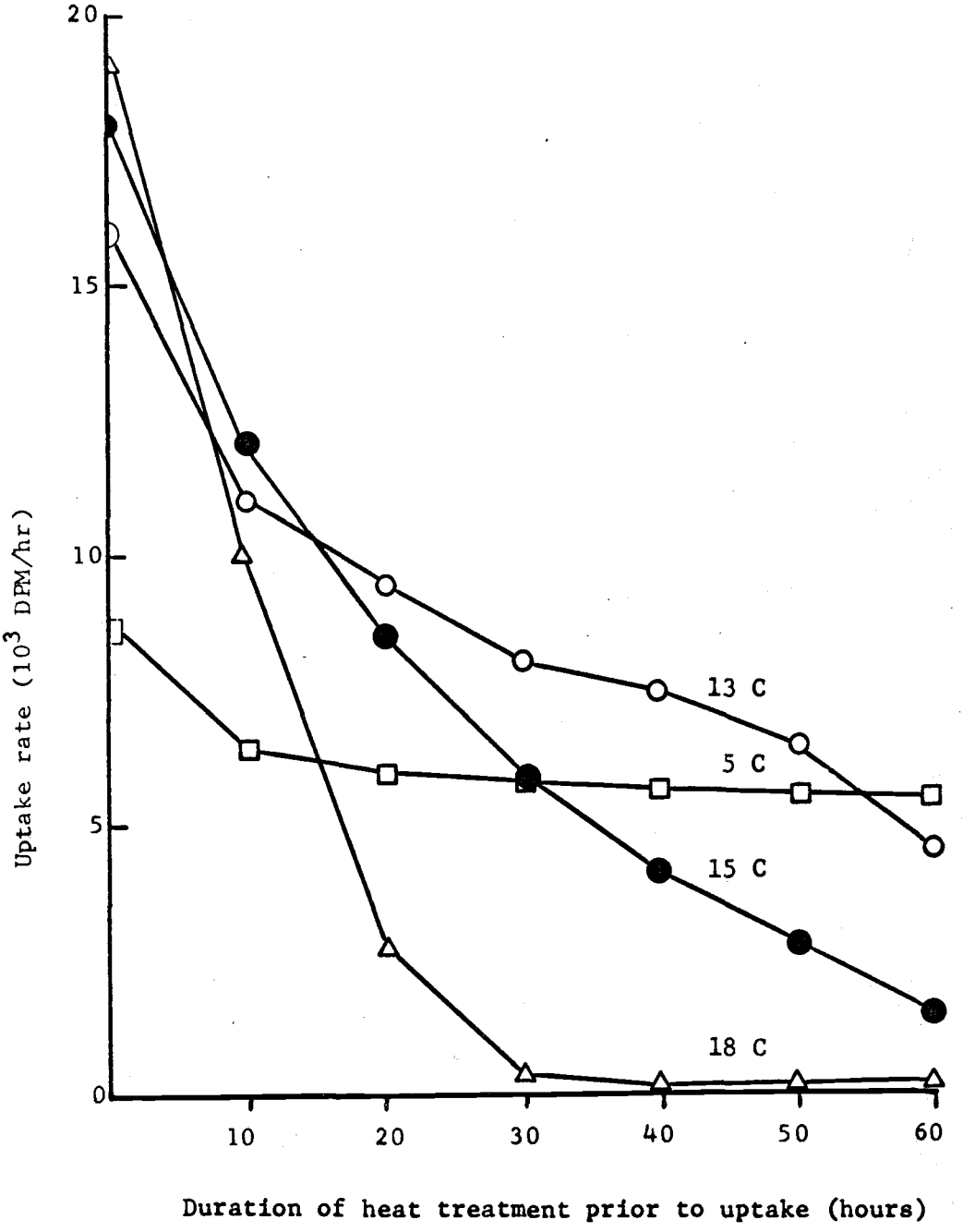

Figure 10. Uptake of ^{14}C-glucose at 5 C (□), 13 C (○), 15 C (●) and 18 C (△) in artificial seawater. Cells were starved of a carbon source for 24 hours prior to the initiation of heat treatment.

ferred to a lower temperature (Fig. 11). The rate of substrate uptake in cells incubated at 13 and 15 C decreased more rapidly with time when the cells were transferred to 5 C than when the rate was measured directly at the incubation temperatures.

The uptake rate of cells heated at 13 C was at all times less than that of the control cells. The rate of cells heated at 15 C was completely inhibited within 20 hours of heat treatment. Therefore, temperatures as low as the maximum growth temperature induced irreversible damage in cells incubated in artificial seawater.

Respiration of Exogenous Glucose During Heat Treatment

During the course of glucose uptake, CO_2 evolution was studied to determine the effects of heating on exogenous respiration. Comparing CO_2 evolution rates in Fig. 12-15 with the respective uptake rates in Fig. 6,7,10, and 11 demonstrates that regardless of the incubation temperature or the type of suspending menstruum employed, $^{14}CO_2$ evolution and the uptake of glucose exhibit similar kinetics.

The irreversible nature of temperature damage in artificial seawater was the same for both the uptake system and for respiration (Fig. 11 and 15). After 60 hours at 13 C, respiration decreased 83 percent from that observed at the onset of heating. At 15 C, a 99 percent decline was noted within the first 30 hours. The result of returning the organism to near optimum temperatures following heat treatment was, therefore, an even greater decrease in respiration. It is important to note that under all conditions considered up to this point, whenever a decrease in respiration

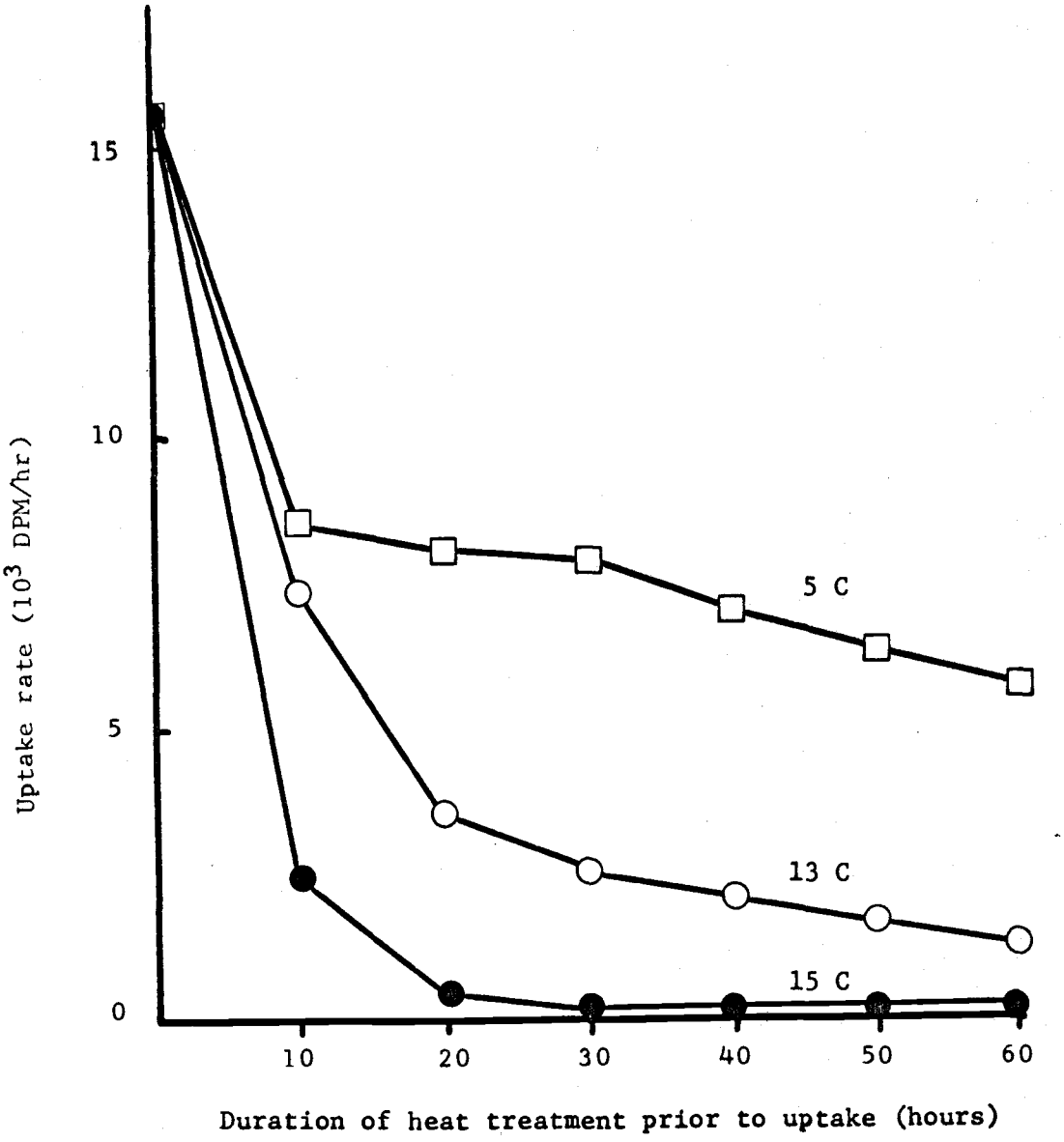

Figure 11. Uptake of ^{14}C-glucose at 5 C after heat treatment at 5 C (□), 13 C (○) and 15 C (●). Cells were starved of a carbon source for 24 hours before the initiation of heat treatment.

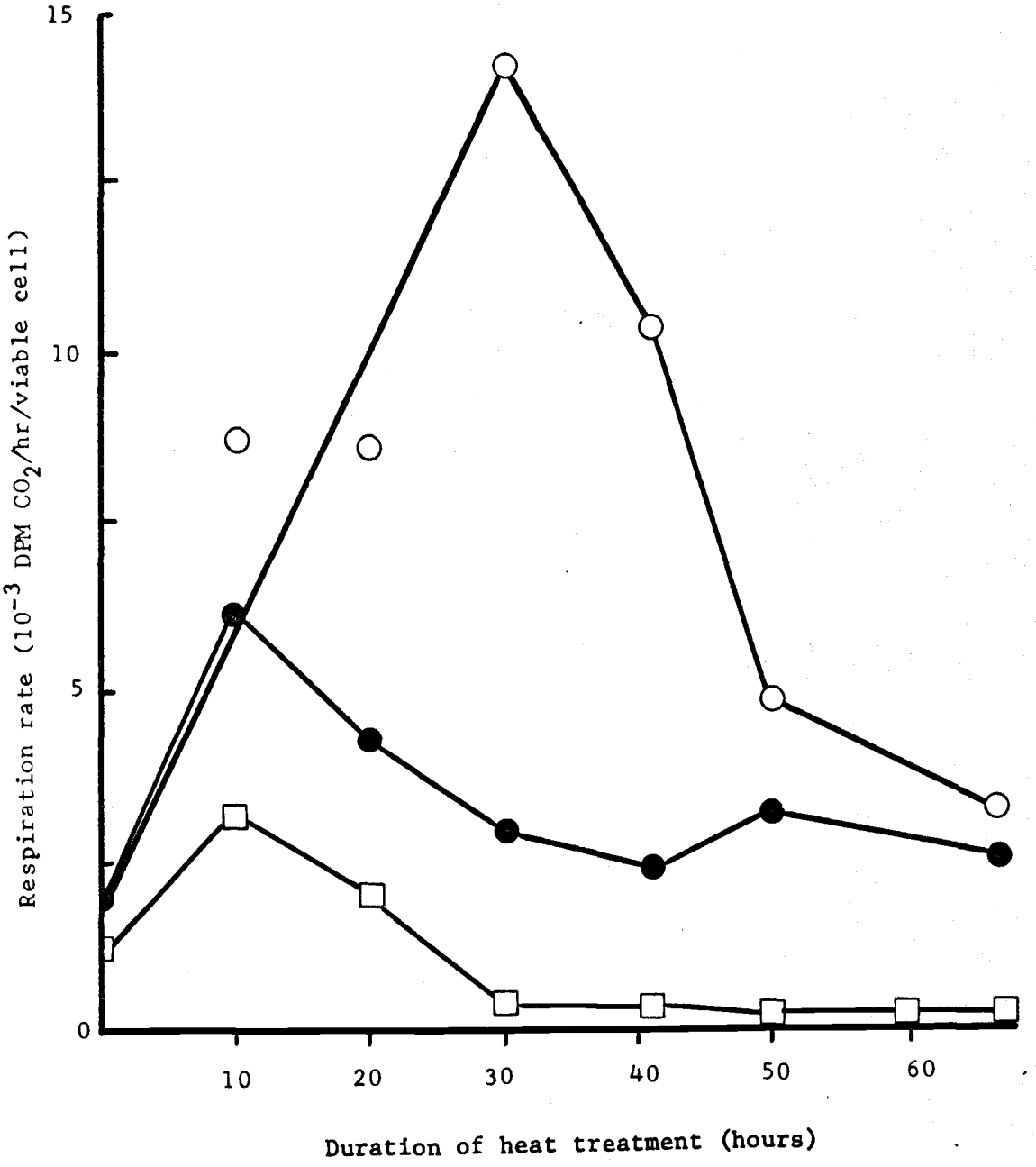

Figure 12. Respiration of exogenous ^{14}C-glucose by cells suspended in complete medium at the heating temperatures of 5 (□), 13 (○) and 15 C (●).

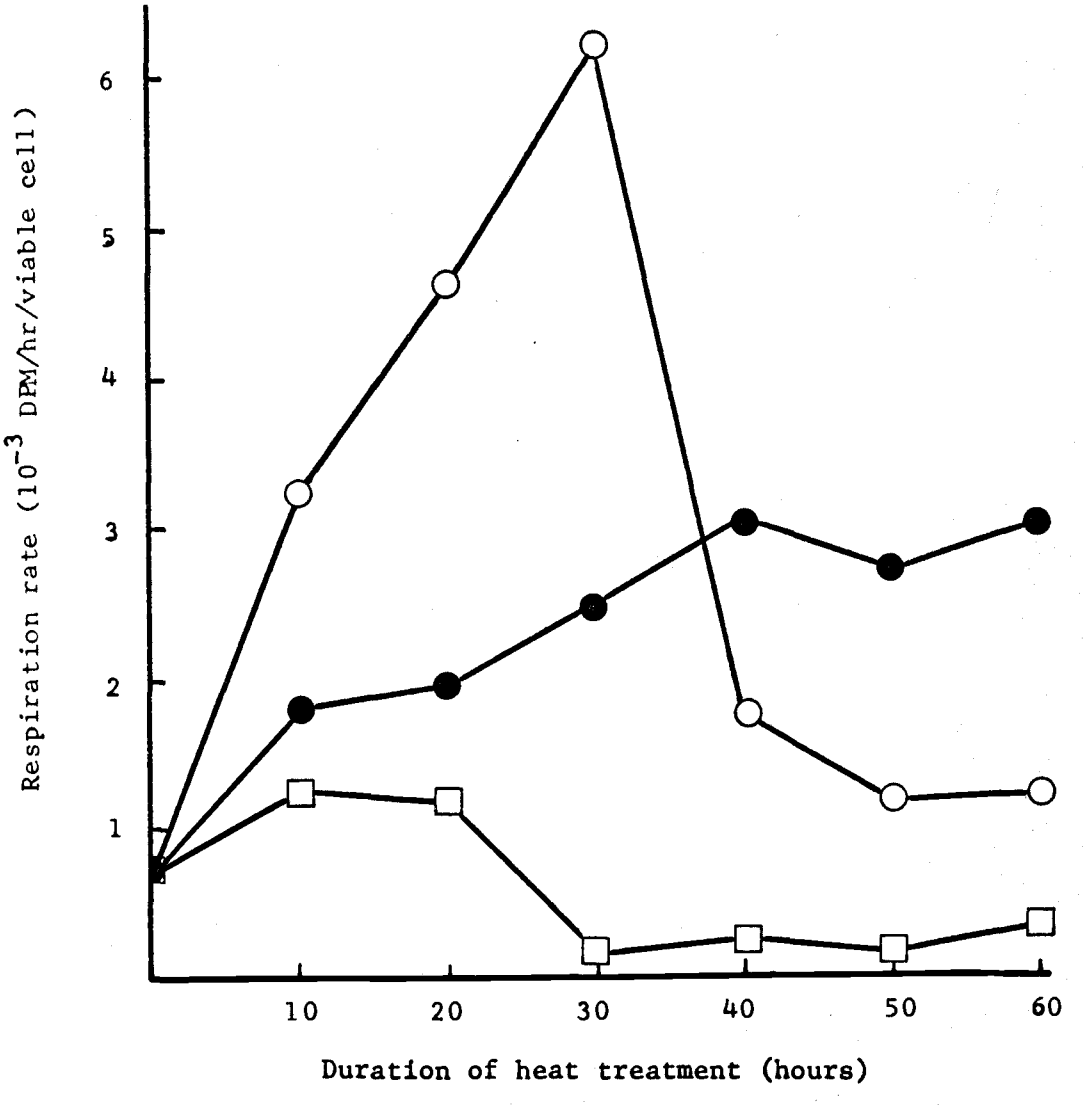

Figure 13. $^{14}CO_2$ evolved from the exogenous utilization of ^{14}C-glucose. The uptake and respiration of ^{14}C-glucose was carried out at 5 C following heat treatment of the cells at 5 (□), 13 (○) and 15 C (●) in complete medium.

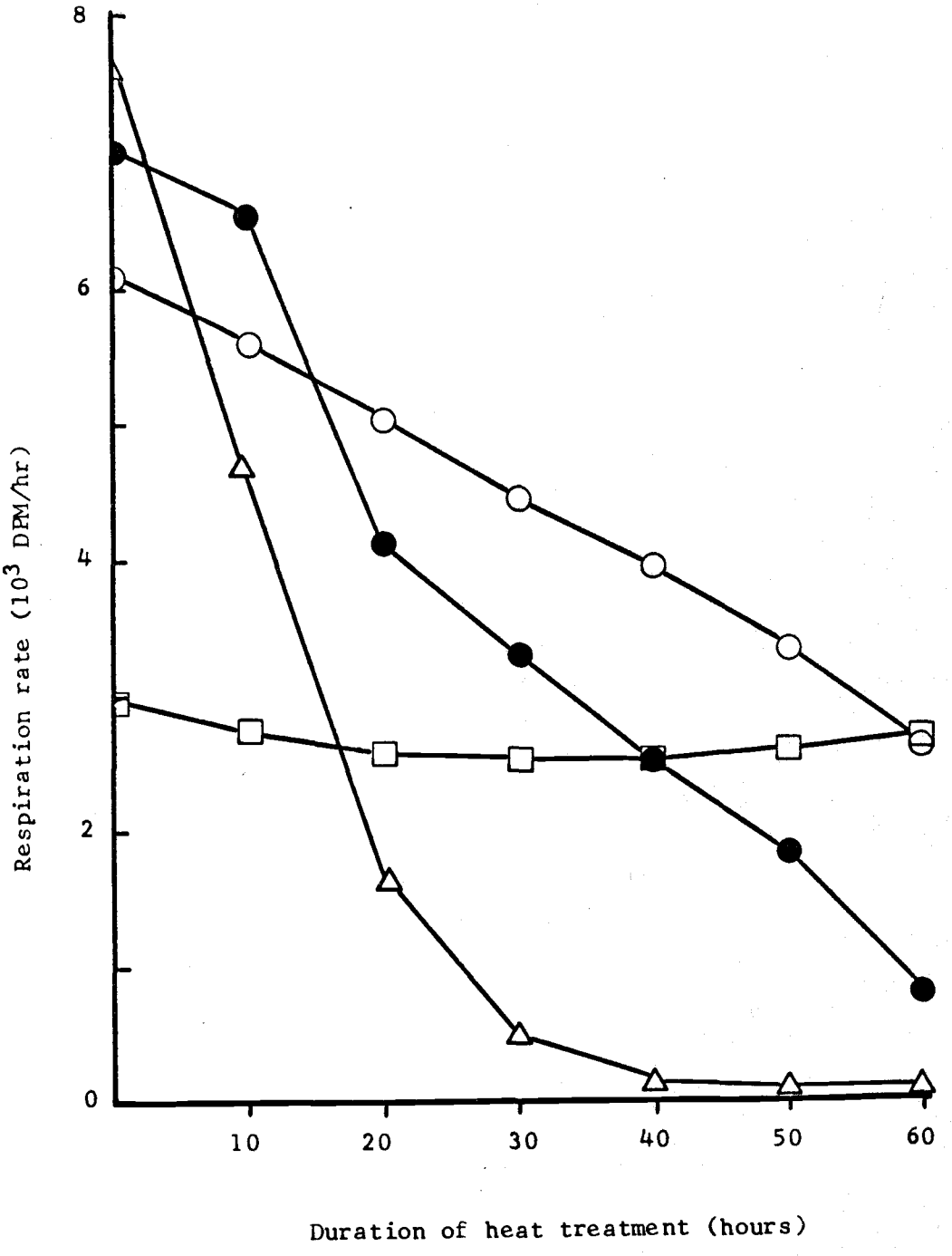

Figure 14. Respiration of exogenous ^{14}C-glucose by cells suspended in artificial seawater at heating temperatures of 5 (□), 13 (○), 15 (●) and 18 C (△). The cells were starved of a carbon source 24 hours prior to initiation of heat treatment.

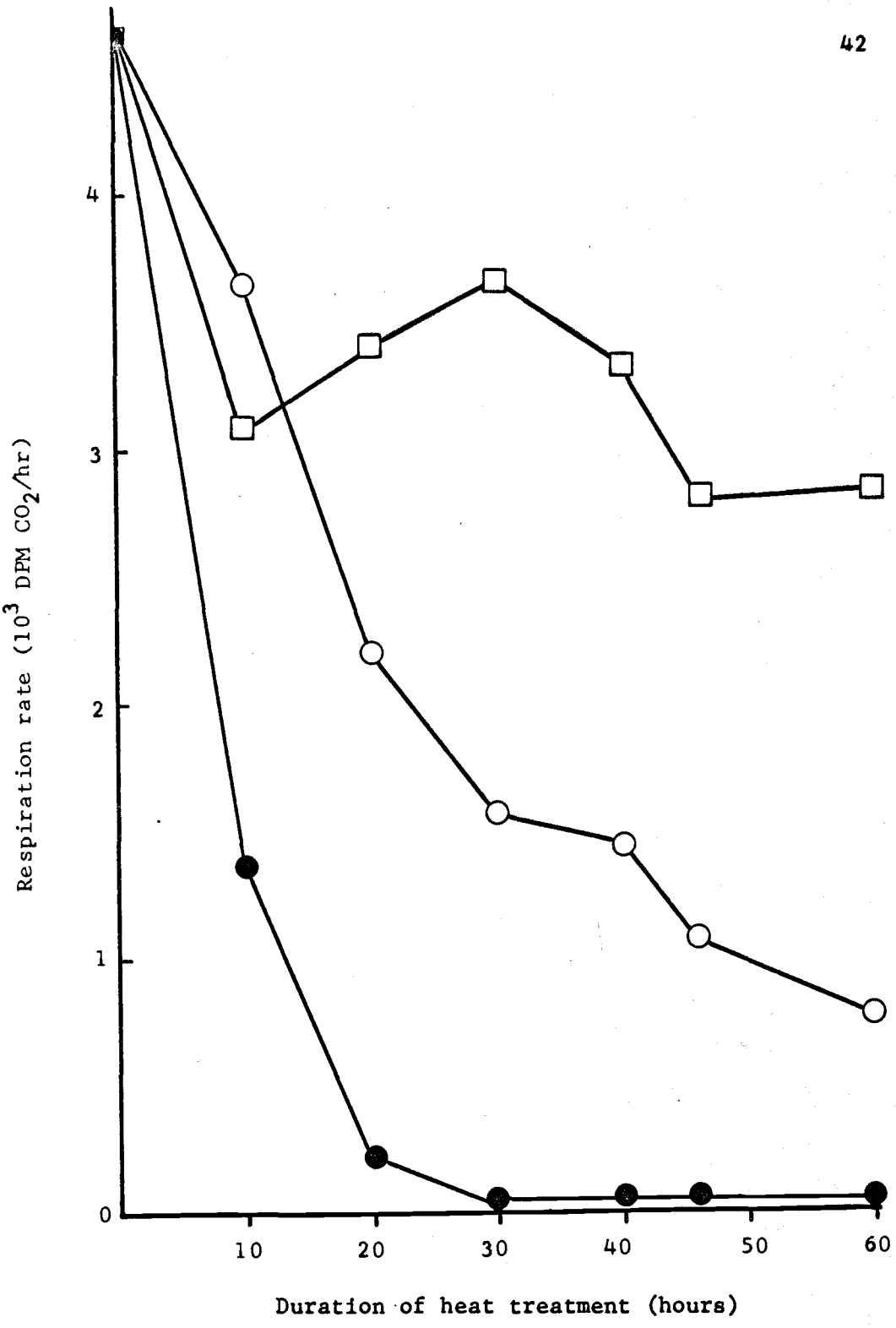

Figure 15. $^{14}CO_2$ evolved from the exogenous utilization of ^{14}C-glucose. The uptake and respiration of ^{14}C-glucose was carried out at 5 C following heat treatment of the cells at 5 (□), 13 (○) and 15 C (●) in artificial seawater.

occurred, there was a concomitant decrease in substrate uptake.

The percentage of carbon taken up that was respired by cells heated in artificial seawater is presented in Table 4. It is apparent that regardless of the temperature, as the duration of heat treatment increased, a greater percentage of the radioactivity associated with the cells after two hours of glucose uptake was respired as $^{14}CO_2$. However, a more extensive increase occurred at 15 C than at 5 and 13 C. A 15 percent increase was observed in the ratio of $^{14}CO_2$ evolved to substrate taken up at 5 and 13 C after 60 hours, whereas at 15 C, a 24 percent increase was noted over the same period.

Endogenous Respiration During Heat Treatment

To isolate the effects of moderate temperatures on respiration, the conversion of endogenous metabolites to $^{14}CO_2$ was studied in pulse-labeled cells. Figure 16 demonstrates that significant changes occurred in the cells between the temperatures of 15 and 18 C as evidenced by $^{14}CO_2$ evolution. Respiration terminated within the first ten hours at 18 C, whereas at temperatures of 15 C or less, $^{14}CO_2$ was evolved at a constant rate for at least 20 hours. The rates at both 13 and 15 C after 20 hours of incubation decreased to approximately eight percent of their initial values shown by the decrease in the slopes of the curves.

Figure 16 also demonstrates that a greater fraction of the radioactivity taken up previously during the pulse-labeling preparation was released as $^{14}CO_2$ at 13 and 15 than at 5 C. This was most apparent during the first 20 hours of incubation.

TABLE 4. RELATIONSHIP OF $^{14}CO_2$ EVOLUTION TO GLUCOSE UPTAKE

Time of heat treatment (hours)	Percent $^{14}CO_2$*		
	5 C	13 C	15 C
0	34	39	40
10	40	51	53
20	41	49	56
30	47	55	56
40	47	54	60
50	51	52	67
60	49	54	64

$^{14}CO_2$ was determined during the 2-hour uptake of ^{14}C-glucose by cells heat treated for various times at 5, 13, and 15 C in artificial seawater.

$$\text{*Percent respired} = \frac{\text{DPM } ^{14}CO_2}{\text{DPM associated with cells} + \text{DPM } ^{14}CO_2} \times 100.$$

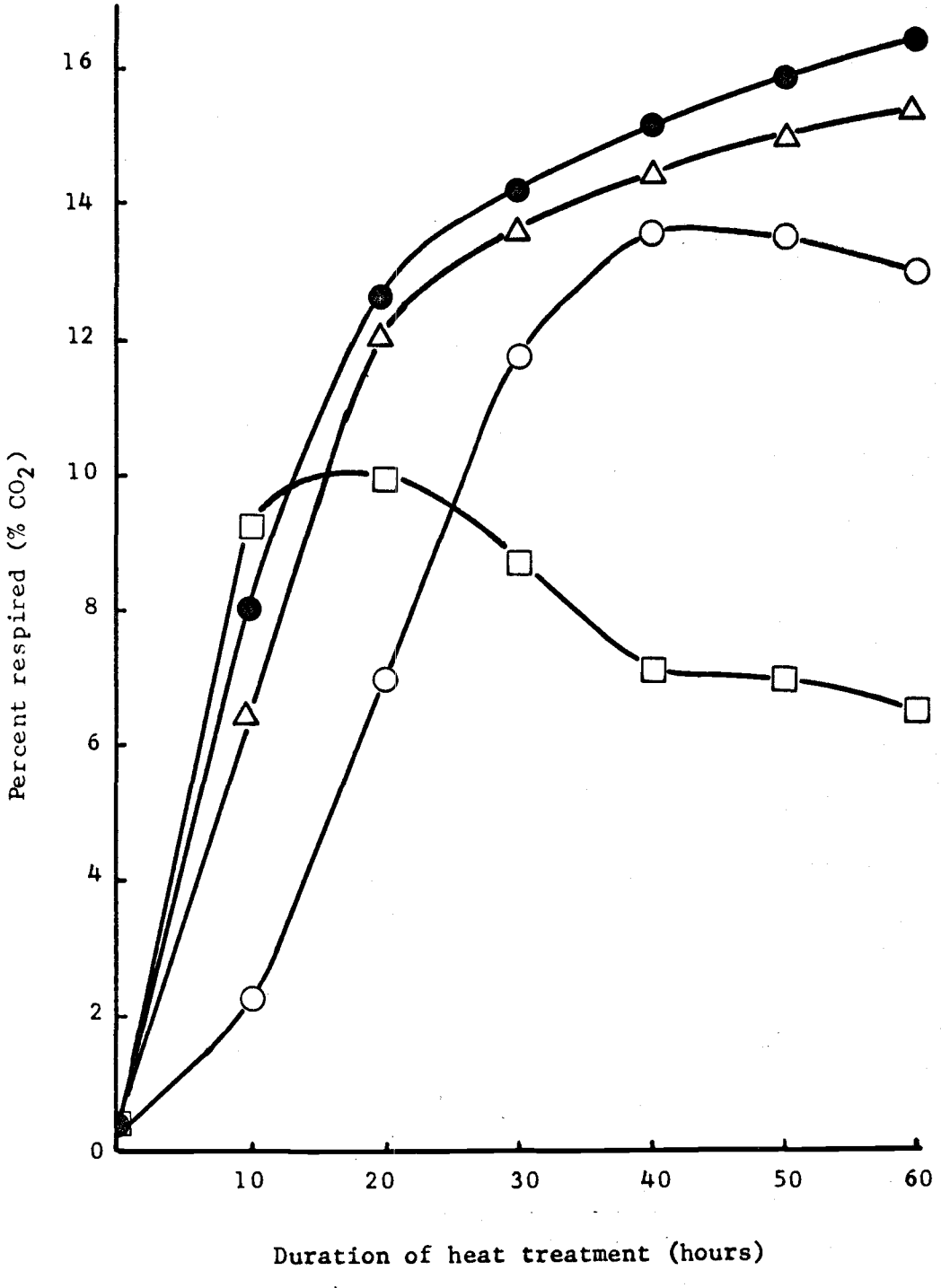

Figure 16. Percent of the radioactivity associated with pulse-labeled cells that is endogenously respired as CO_2 during heat treatment at 5 (○), 13 (△), 15 (●) and 18 C (□) in artificial seawater.

DISCUSSION

By definition, Ant-300 is an obligate psychrophile. Greater cell yields obtained with prolonged incubation at low temperatures typify the cold-loving nature of the organism. It is likely that the more efficient use of nutrients by the cells at lower temperatures resulted from increased O_2 solubility.

These studies indicate that at least two membrane associated functions appeared to undergo heat damage at temperatures close to the maximum growth temperature. Glucose uptake seemed inhibited at 13 C and control of leakage was lost at 15 C. The results are consistant with the views of D Aoust and Kushner (1971) that the fine structure of the outer layers of marine psychrophiles is extremely susceptible to environmental changes.

Permeability control, as expressed through leakage, exhibited extraordinary heat sensitivity. Minimum leakage was demonstrated at temperatures near the optimum growth temperature (5 C). Much of the leakage material at this temperature was most likely labeled glucose released from the outside of the cell by acid treatment (Hobbie and Crawford, 1969). The increased leakage noted at higher temperatures is consistant with the results of Alsobrook et al. (1972) who observed that increasing temperatures induced the release of increasing amounts of protein and RNA from Bacillus psychrophilus. They noted that leakage occurred at temperatures at which viability was maintained. A comparison of Fig. 3 and 4 of this study also illustrates that during the first ten hours of heat treatment, while

leakage was extensive, the cells remained viable.

Kenis and Morita (1968), on the other hand, observed leakage of cellular material at temperatures no less than 5 C above the maximum growth temperature of V. marinus MP-1. In addition, leakage was not evident until all but 0.2 percent of the cells were dead.

The apparent differences regarding the onset of leakage may be related to the techniques employed for detecting the leakage material. It may be recalled that in this study insignificant amounts of leakage of DNA and RNA were detected with the diphenylamine and orcinol procedures used by Kenis and Morita (1968). Only by means of the sensitive isotopic tracer technique was leakage detected at low temperatures.

An alternative explanation for the delayed release of material from V. marinus MP-1 may be that the rich nutrient medium in which the cells were suspended provided some protection to the membrane during heat treatment. The effects of nutrients on permeability control, however, were not considered in the present investigation.

In these studies it was observed that leakage of material eventually stopped at temperatures up to and including the maximum growth temperature. However, at temperatures above the maximum growth temperature, release of cellular material was continuous. The close correlation between the maximum growth temperature and the temperature at which leakage became continuous implies that growth may be limited by the prolonged leakage of intracellular metabolites.

It has been proposed that lysis contributes to the accumulation of cellular material in the heating menstruum (Kenis and Morita, 1968). The following evidence suggests that lysis did not occur in Ant-300 at 15 C, the temperature at which leakage was continuous and viability decreased. First, CO_2 evolution at 13 and 15 C were similar (Fig. 4). Therefore, although a decrease in viability took place at 15 C (Fig. 3), the respiratory system remained intact. Secondly, lysis did not occur at 18 C after four hours of incubation during which extensive leakage had occurred (Fig. 5). It is likely then, that although leaky cells did not retain 100 percent viability on SDB agar medium at 5 C following prolonged heating at 15 C, they did not lyse.

The views of Haight and Morita (1966) that the release of specific cellular components may effect the inactivation of psychrophilic bacteria at elevated temperatures gained support from this study. The recovery of non-dialyzable radioactive leakage material from heat treated cells (Table 2) implies that significant quantities of macromolecules were release prior to death or lysis.

Further investigation of the nature of the leakage components indicated the presence of protein and RNA; protein was present in greater quantities than RNA.

The increased release of RNA by cells at elevated temperatures supports the observations of Iandolo and Ordal (1966) and Gronlund and Campbell (1965). These results are also consistent with those of Strange and Shon (1964) and Sogin and Ordal (1967) that the release of RNA precedes bacterial death.

DNA and free amino acids were not present as a measurable portion of the leakage material. Previously, Haight and Morita (1966) and Kenis and Morita (1968) demonstrated the presence of these cellular constituents in the leakage fraction of V. marinus MP-1. The low concentrations of cells (approximately 2 x 10^6 cells/ml) employed in these studies may have been responsible for the difficulty in recognizing the presence of these components in the heating menstruum.

Alternatively, the possibility exists that membrane alterations at 18 C are distinct from those changes observed at 25 C in V. marinus MP-1. These distinctions may be illustrated by the types of compounds that leak out of the cell.

Substrate uptake in bacteria has been shown to be quite sensitive to elevated temperatures. Kaback (1970) demonstrated in E. coli that with increasing temperatures the level of α-methyl glucoside uptake reached a maximum at 40 C and then declined rapidly. Using a psychrophile in these studies, it was found that glucose uptake was restricted at the maximum growth temperature of 13 C under conditions in which the cells were suspended in artificial seawater. It was further demonstrated that the uptake restrictions imposed by 13 C temperatures were not removed after the cells were transferred to 5 C. At 13 C, the reduced accumulation of radioactivity by the cells after 20 hours was not the result of leakage since the release of intracellular material had stopped within this period (see Fig. 4). Despite the inhibited glucose uptake, 100 percent viability was observed when the organisms were transferred to a complex medium

containing other utilizable carbon sources and incubated at 5 C.

Higher temperatures produced a more rapid and extensive inhibition of uptake as indicated in cells incubated at 15 and 18 C. It was difficult to determine at those temperatures, though, whether the decreased accumulation of radioactivity resulted from increased heat damage to the glucose uptake mechanism. Quite possibly the decreased accumulation resulted from leakage of the substrate back into the menstruum. At 15 and 18 C, the cells did not remain viable for prolonged periods in artificial seawater.

Kay and Gronlund (1969) concluded that the transport of the amino acid, valine, could be dissociated from growth since the initial rate of substrate uptake was high at 45 C, whereas growth of the organism, Pseudomonas aeruginosa, did not occur at this temperature. However, the organism was heated for a relatively short period of time before uptake studies were initiated. Similar results were obtained after short periods of heating Ant-300. Uptake of glucose was initially high at 18 C (a temperature at which growth did not occur). Not until after prolonged incubation at low temperatures was there a significant reduction in the uptake of glucose. Thus, like any enzyme catalyzed reaction, it is important to consider the duration of heating when determining the heat sensitivity of cellular mechanisms.

The presence of nutrients appeared to have some beneficial effect on glucose uptake at near maximum growth temperatures. The addition of 0.25 percent glucose and 0.05 percent yeast extract allowed the uptake rates in cells incubated at 13 and 15 C to remain above the

5 C control over the entire 60 hour investigation (Fig. 6). It may be recalled in Fig. 10 in which cells were suspended in artificial seawater that uptake at 13 and 15 C dropped below the uptake rate at 5 C after 50 and 30 hours, respectively.

The addition of nutrients also increased the viability of cells heated above the maximum growth temperature (i.e., 15 C). Haight and Morita (1966) similarly noted that the viability of V. marinus MP-1 was increased during heat shock at 25 C when organic nutrients were present. However, the precise nature in which the nutrients protected the cells was not determined. The results of these studies suggest that the increased temperature tolerance of Ant-300 in complete medium may be related to the protection that nutrients conferred directly upon the glucose uptake mechanism.

The uptake pattern displayed at 5 C in cells heated at 13 and 15 C, shown in Fig. 7, suggest that the rate of glucose uptake may be influenced by leakage. The uptake rate in cells heated at 13 C dropped below the rate of uptake in cells heated at 15 C after 30 hours. The cells heated at 13 C may have assumed the low uptake rate in response to the cessation of leakage at that temperature after 20 hours. Cells in which leakage was continuous, as was demonstrated at 15 C, would be expected to maintain a higher rate of substrate uptake after 20 hours in order to replace the material lost across the membrane.

It is difficult to explain, though, why uptake in cells heated at 13 C did not drop below that of cells heated at 15 C when uptake was measured directly at the heating temperatures shown also in Fig. 7. The inconsistancy may result from the growth phase differences

encountered at 5, 13, and 15 C during heat treatment in complete medium.

In the presence of nutrients, glucose uptake appeared to be related to the phases of cell growth. The rate of glucose uptake reached a maximum at the end of lag phase and decreased during log growth. This observation is in agreement with the idea that metabolic activity attains its maximum at the end of the phase of accelerated growth (Burrows, 1968). It was difficult, therefore, to compare the rates of uptake of glucose among cultures with different growth characteristics. Presenting the rate of uptake on a "per cell" basis where growth occurred during heat treatment seemed the most reasonable means for comparing the uptake of glucose among cells held at different temperatures.

Robison and Morita (1966) noted in V. marinus MP-1 that at temperatures less than 1 C above the maximum growth temperature, both exogenous respiration from glucose and endogenous respiration decreased with time of heating. It was suggested that heat labile respiratory enzymes were responsible for the observed decrease.

The decreased respiratory activity that occurred in Ant-300 when the temperature was increased from 13 to 15 C was not likely due to the inactivation of heat labile respiratory enzymes. Studies on endogenous respiration (Fig. 16), demonstrated that respiratory activity at 13 and 15 C was quite similar.

Instead, the decreased exogenous respiration at 15 C may have resulted from a limited availability of substrate within the cell. Evidence of this was noted in the kinetic similarities between

^{14}C-glucose uptake and subsequent respiration as $^{14}CO_2$ by cells heated at 13 and 15 C.

The increase in percent CO_2 respired with time at 5 C, illustrated in Table 4, indicates that there is decreasing participation of endogenous reserve material and increasing participation of exogenous glucose in cellular maintenance. The decrease in endogenous reserve material suggests that the cells were undergoing starvation. Starvation might be expected as the cells were suspended in artificial seawater for a relatively long period of time. The higher percent CO_2 at 13 and 15 C also noted in Table 4 support the findings of Allwood and Russel (1967) that starvation is accelerated by increasing temperatures.

Temperature accelerated starvation is also illustrated in Fig. 16. Endogenous $^{14}CO_2$ evolution decreased from the initial rate more rapidly at 13 and 15 C than at 5 C illustrated by the decrease in the slopes of the curves. The cessation of respiration at temperatures as high as 18 C, however, may involve more than starvation.

It is quite possible that the decreased respiration of endogenous material observed by Robison and Morita (1966) was also the result of a starvation process accelerated by temperature. Within limits, inactivation of specific respiratory enzymes would not be the cause of the decreased respiratory activity at elevated temperatures. Whether the respiratory system was at all damaged by 13 and 15 C temperatures remains to be determined.

The data suggest that the growth of Ant-300 at moderate temperatures was restricted by the heat inactivation of the glucose uptake

mechanism in addition to a breakdown of permeability control across the membrane. The greater turnover rates of cellular components at high temperatures, demonstrated by the quantity of $^{14}CO_2$ evolved, increased the energy requirements for maintenance. The maximum growth temperature occurred at a temperature where the uptake of substrate was restricted to the extent that most of the available intracellular energy was directed to maintenance and only limited energy was available for growth. At temperatures slightly above this (i.e., 15 C), where greater inhibition of substrate transport and continuous leakage occurred, the energy requirements for growth were not met. Under these conditions the cell acquired only enough energy to remain viable.

The maximum temperature for viability was greater than the maximum temperature for growth. Hence, the physical and chemical effects which prevented growth of the organism may not necessarily be related to those effects determining their death.

Lysis is one event that does not appear to have occurred at the maximum growth temperature but has been considered in relation to the death of some organisms (Kenis and Morita, 1968). Further investigations concerning the relationship between lysis and death should be considered before the impact of temperature sensitive uptake, leakage, and respiration can be determined in terms of the ultimate survival of the organism at elevated temperatures.

SUMMARY

The results of this study indicate that several membrane associated functions of the marine psychrophile, Ant-300, exhibited heat sensitivity at temperatures as low as the maximum growth temperature. Loss of permeability control as expressed by leakage of intracellular material occurred at the maximum growth temperature of 13 C and became more extensive and continuous at temperatures above this. Determination of the leakage material indicated that macromolecules in the form of protein and RNA were released from the cells. The glucose transport system appeared to be restricted at the maximum growth temperature, also. However, the activity never became completely inhibited over the extended time period. Increased temperatures seemed to accelerate both the rate and extent of inactivation of uptake. The presence of nutrients afforded some protection to the uptake system against heat injury. Respiration seemed to be less heat sensitive than either of the two membrane associated functions. It was suggested that the rate of substrate metabolism and CO_2 evolution is regulated more by the availability of substrate than by a heat sensitive respiratory enzyme. It was further suggested that heat sensitivity disturbs the normal functions associated with the cell membrane in a manner by which energy becomes limited inside the cell. As a result, the energy normally utilized for growth is diverted toward cell maintenance, allowing viability at moderate temperatures but restricting growth. Further restrictions on these same functions

may ultimately bring about the death of the organism at slightly higher temperatures.

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