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Title STUDIES ON THE EFFECT OF MODERATE TEMPERATURE ON VIBRIO MARINUS

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Washed cells of Vibrio marinus MP-1 grown at 15 C (organism's optimum growth temperature) were employed in this study. These cells were exposed to heat at and above their maximal growth temperature (20 C) for one hour and tested manometrically at 15 C for their ability to take up oxygen.

Endogenous activity, as evidenced by oxygen uptake, decreased with increased temperatures of exposure and was destroyed at 30 C. The same pattern of oxygen uptake was noted when glucose was employed as the substrate. Oxygen uptake decreased with increasing exposure periods at temperatures above the maximum growth temperature. Endogenous oxygen uptake was negligible after exposure at 29.2 C for 50 minutes.

The supernatants, which resulted from suspensions of cells exposed to 15 to 35 C, contained 260-280 mμ absorbing material. This increased with increased temperature as well as increased time of

exposure. The most radical changes occurred above 28 C. Supernatants were further analyzed for Kjeldahl nitrogen, orcinol reacting material and diphenylamine reacting material. The latter are indicative of ribonucleic and deoxyribonucleic acid respectively.

The data indicates that moderate temperatures from 20 to 30 C are sufficient to inactivate the metabolic systems involved in oxygen uptake both endogenously and in the presence of glucose. Also, cellular composition and permeability are affected, as evidenced by the leakage of 260-280 mμ material.

STUDIES ON THE EFFECT OF MODERATE TEMPERATURE ON VIBRIO MARINUS

by

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In appreciation to all those whose

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STUDIES ON THE EFFECT OF MODERATE TEMPERATURE ON VIBRIO MARINUS

INTRODUCTION

It is recognized that over 90 percent of the marine biosphere has a temperature below 5 C (13, p. 110). Most marine organisms are killed at temperatures above 30 C (72) indicating the existence of some unique features to maintain the physiological balance necessary for cellular integrity at low temperatures.

Many forms of life in the marine environment require cold temperatures for survival. At these low temperatures, they make a notable contribution to geology and play an important role in the cycles of matter in the sea. As a contributing member, the microbe can give an indication of the systems limiting certain forms of life to low temperature.

Psychrophily is not limited in importance only to the marine environment. Many environments and industries utilize low temperature. The growth of microorganisms at low temperatures constitutes a major problem, especially in the fields of food, dairy and pharmacy. Even if an organism is unable to grow, it may possess enzymes in a suitable environment to degrade the product. Not only is the viable cell important, but also the whole cell as a site for synthesis of enzymes and enzymatic activity.

Stokes (60) defined psychrophilic bacteria as those which grew sufficiently at 0 C to become macroscopically visible in about a week. Psychrophilic bacteria were subdivided into obligate or strict psychrophiles and facultative psychrophiles depending on their ability to grow rapidly below or above 20 C. His definition will be employed throughout this paper.

Recognizing that the maximum growth temperature of Vibrio marinus MP-1 is 20 C (42), some rational explanation of this phenomenon is needed. Proteins, especially enzymes, are known to be one of the most heat labile components of cells. Recognizing this fact, the question of the effect of moderate temperatures of 20 to 30 C on the oxidation of glucose by MP-1, an obligate marine psychrophile, was initiated using manometic technique. Such studies would indicate the possible existence of thermolabile enzymatic reaction(s) involved in the oxidation of glucose. This would also help to explain the obligate psychrophilic nature of V. marinus MP-1 and the low temperature maximum of biological systems from low temperature environments.

Simultaneous observations of the density of cell suspensions, nitrogenous materials in the supernatant and viability were carried out to relate the effect of temperature to the cells integrity as a viable entity.

REVIEW OF LITERATURE

Vibrio marinus MP-1 is an obligate marine psychrophile. This organism has an optimum growth temperature of 15 C. It does not grow above 20 C (42). The organism has been described taxonomically by Colwell and Morita (15).

Morita and Haight (42) have studied the thermolability of MP-1 in media. These studies indicate that the organism after exposure to 25 C for nine hours retained ability to repair cellular damage caused by heat. Reversible damage also occurred after three hours exposure at 28.8 C and 30.8 C as indicated by prolonged lag periods. Viability was destroyed after 6.25 hours.

Burton and Morita (11) and Morita and Burton (41) demonstrated loss of permeability control as well as heat denaturation of malic dehydrogenase at moderate temperatures with cells of another strain of V. marinus, a facultative psychrophile. Malic dehydrogenase activity in lysed cells was 12 times that of whole cells. The enzyme was more sensitive to heat when cells were lysed. This would indicate that the cell membrane may play a vital role in maintaining the physiological balance necessary for cell integrity.

Bedford (4) observed that the temperature range of growth for marine bacteria from the north Pacific Ocean ranged from -7.5 to 45 C. With the exception of four all grew well at 0 C. Hess (26)

reported maximum cell yields of three marine species at 5 C. ZoBell (71) observed that most marine bacteria reproduce at 0 and -4 C. ZoBell and Conn (72) reported that 30 C for ten minutes killed 25 percent of the bacteria from marine sediments and sea water. Only 20 percent remained viable after ten minutes at 40 C. They also reported a decrease in oxygen utilization by heat treated marine bacteria at temperatures of 30 C and above.

Until very recently research into the nature of psychrophily has been limited to facultative psychrophiles. This is, in part, due to the temperatures incurred in handling and processing samples. Even so, numerous contributions have been made in attempting to explain the mechanisms involved in biochemical activities and life at low temperature. Several excellent reviews have been published by Berry and Magoon (5), Ingraham and Stokes (31), Rose (57) and the Campbell Soup Company Low Temperature Microbiology Symposium (13).

The rates of oxidation by psychrophiles are affected less by a decrease in temperature than those of mesophiles (8, 23, 30, 31). Ingraham and Bailey (30) found little difference in oxygen uptake of cell free extracts of mesophilic and psychrophilic organisms. Brown (8) noted in his studies that glucose oxidation occurred at temperatures considerably above the optimum growth temperature of the psychrophile. Lowering the temperature did not affect the growth requirements of the organisms he observed.

Several workers including Lichstein and Begue (36) and Borek and Waelsch (7) have shown variations in nutritional requirements with temperature. The effect of temperature on fatty acid composition of psychrophilic and mesophilic species of Saccharomyces (36), Serratia (33) and Candida (32), as well as Escherichia coli (40) and marine ectotherms (37) have been observed. Sultzer (64) has noted differences in oxidation in the presence of fatty acids in Serratia marcescens and Sarcina flava.

The growth, survival, respiration of intact cells, alcohol dehydrogenase activity and entry of glucosamine into psychrophilic Candida were more sensitive above 10 C than a corresponding mesophile (3). Cirillo, Wilkins and Anton (14) reported a similar effect involving sugar transport.

Upadhyay and Stokes (66), working with a facultative psychrophile, demonstrated that formic hydrogenlyase was inactivated at 45 C and that formic hydrogenlyase synthesis was inactivated at 20 C. They have also found inactivation of hydrogenase at 20 C, while hydrogenase activity was reduced 50 percent at 60 C after two hours (67).

Azuma and Witter (2) working with a mesophile and UV produced psychrophilic mutant of the mesophile showed that for both organisms the lower the growth temperature, the more fragile the protoplasts. At the same temperature the mesophile was more fragile than the psychrophile.

Recently Hagen, Kushner and Gibbons (23) have reported a prodigious producing rod which will not grow at 21 C. Viable counts of cells suspended in sea water were reduced 1000 fold after 30 minutes at 25 C. Ninety percent of the population was nonviable before a decrease in turbidity was observed. A decrease in lipid phosphate occurred at 25 and 35 C and a phosphatidase was noted in cell lysates. This bacterium is the only other obligate psychrophile reported in the literature to date.

Endogenous metabolism encompasses "all the chemical activities performed by organisms in the absence of utilizable extracellular substrate" (34). Mallete (38) stressed the importance of endogenous metabolism in relation to the natural environment which may be temporarily devoid of nutrients. Dawes and Ribbons (16) emphasized that the significance is limited to the viable cell. The relationship to survival and the effect of environmental conditions on endogenous metabolism are now being investigated by a number of workers.

Changes in the macromolecular composition of endogenously respiring cells have been observed to be accompanied by release of UV absorbing materials into the suspending medium (9, 12, 22, 25, 27, 28, 37, 39, 45, 46, 54, 56, 59, 61, 62, 65, 68). This phenomenon is apparently not limited to endogenously metabolizing cells.

Okabayashi, et. al. (47, 48, 49, 50, 51, 52) have extensively studied nucleotide leakage in the culture fluid of Brevibacterium liquefaciens.

MATERIALS AND METHODS

Growth Medium

Rila Marine Mix (Rila Products, Teaneck, N. J.), referred to as sea water, was employed in a concentration of 26. 25 g/liter distilled water.

Medium 2216 E contained: 5. 0 g neopeptone (Difco), 1. 0 g yeast extract (Difco), 0. 1 g ferric phosphate and one liter sea water. Where designated 0. 5 g glucose/liter was added to the medium. The pH was adjusted with 1 N NaOH. The pH of the medium after autoclaving was 7. 25. The medium was dispensed in 15 x 120 mm screwcap test tubes in 7 ml portions or into wide mouth Fernbach flasks in 300 ml portions. Sterilization was effected by autoclaving 20 minutes at 15 psi.

Prior to inoculation all media was cooled to 15 C or below.

Stock Cultures

Stock cultures were maintained on 2216 E medium in screwcap test tubes in the refrigerator and transferred at least once a month. A stock culture was maintained at 15 C and transferred daily for at least one week prior to preparation of the inoculum.

Preparation of Cells

The inoculum was prepared in screwcap test tubes containing 2216 E + glucose from a 15 C stock culture of V. marinus MP-1. Two transfers were made at 12 hour intervals prior to inoculating each Fernbach flask containing similar media, with four ml. The flasks were incubated at 15 C in an incubator shaker (New Brunswick Psychro-Therm) with a reciprocating mechanism at 120 strokes per minute and a 2.54 cm stroke for 12 hours. At this time the culture was observed to be in the late logarithmic growth phase.

All steps in harvesting cells were carried out on ice or at 0 C. The culture was centrifuged at 5,000 x g in a Sorvall RC-2 Super-speed refrigerated centrifuge for 10 minutes and washed once in sea water.

Cells for Warburg studies were suspended in a 500 ml Erlenmyer flask with sea water to a volume of 25 to 30 ml per original culture flask. Cells were aerated by shaking at 15 C for 70 minutes in the New Brunswick Psychro-Therm. The cell suspension was then centrifuged at 8,000 x g and suspended in sea water. The turbidity was adjusted so that a 1:100 dilution of cell suspension gave an optical density (OD) of 0.220 $\pm$ 0.01 at 600 mμ using one cm cuvettes in the Bausch and Lomb Spectronic 20 spectrophotometer.

Cells for leakage and permeability studies were suspended to

the desired OD without aeration or subsequent washing.

If viability studies were to be made, all equipment was sterilized and aseptic technique employed.

A portion of the cell suspension for Kjeldahl analysis was frozen.

Temperature Exposure

Sterile 15 x 150 mm test tubes with metal caps were equilibrated in constant temperature water baths or a polythermostat (53). Portions of 4. 5 ml were delivered into the test tubes at zero time or suitable intervals. All tubes were placed simultaneously on ice for 15 minutes following heat treatment.

Viability Studies

Dilution blanks were prepared using one g proteose peptone (Difco) per 1000 ml sea water and pipetted after sterilization into 15 x 100 mm sterile screwcap test tubes. The growth medium was 2216 E + glucose.

All media, pipettes and experimental procedure were at 8 to 10 C. Most Probable Numbers (MPN) were estimated for endogenous Warburg samples using tenfold dilutions with replicate aliquots of one ml sampled from each dilution (1). Results were recorded after three days incubation.

Manometric Studies

Oxygen uptake was determined at 15 C using standard Warburg manometric technique.

The 0. 2 M tris-phosphate-salts buffer was prepared as follows: 5. 34 g sodium chloride, 0. 18 g magnesium chloride, 0. 75 g sodium sulfate, 0. 12 g potassium chloride and 6. 05 g tris-(hydroxymethyl)-aminomethane (Tris), were dissolved in 150 ml deionized distilled water. Dilute phosphoric acid was added to a pH of 7. 25. The buffer was brought to 250 ml with deionized distilled water.

The total Warburg flask volume of 3. 2 ml consisted of 1. 8 ml 0. 2 M tris-phosphate-salts buffer, 1. 0 ml cell suspension, 0. 2 ml glucose solution (one μmole) or distilled water in the side arm and 20 percent KOH in the center well.

Cell suspensions were cooled on ice for 15 minutes. Delivery of the cells into chilled Warburg flasks and assembly of the manometers took 30 minutes. The flasks were equilibrated for 15 minutes.

Preparation and Analysis of Supernatant

Following heat treatment, the cell suspensions were immediately transferred to chilled centrifuge tubes and centrifuged at 12, 000 x g for 20 minutes at 0 C The supernatant samples for Kjeldahl nitrogen determinations were frozen.

Samples were analyzed in a Beckman DU spectrophotometer using 1.0 cm cuvettes. Protein and nucleic acid estimations were based on the extinction coefficient of enolase and nucleic acid given by Warburg and Christian (69).

The DNA estimation was carried out directly on the supernatant fluid using a modification of the procedures of Burton (10). The amount of diphenylamine reacting material was determined spectrophotometrically with the Bausch and Lomb Spectronic 20 at 600 mμ and recorded using salmon sperm DNA Type III (Sigma) as a standard.

The RNA estimation was also carried out directly on the supernatant fluid by the method of Schneider (58) using orcinol. Results were read on the Spectronic 20 at 660 mμ and recorded in terms of yeast RNA (Sigma).

Micro-Kjeldahl Determination of Nitrogen

The digestion mixture consisted of 150 mg anhydrous $CuSe0_4$, 144 ml concentrated H_2SO_4 brought to one liter with deionized distilled water.

Total nitrogen was determined after three hours digestion by direct Nesslerization. The color was read at 480 mμ using a Bausch and Lomb Spectronic 20 colorimeter.

RESULTS

Manometric Studies

Figure 1 shows oxygen uptake in the presence of glucose. Values were corrected for endogenous activity. Oxygen uptake decreased with increased time of exposure to 29.4 C. To a great extent this probably reflected death of cells resulting from thermal damage to the cell at 29.4 C. No oxygen uptake could be noted after exposure to 29.4 C for 30 minutes. The number of viable cells decreased from 10^{10} to 10^{4} (Figure 8) after heating for 43 minutes.

Oxygen uptake in the presence of glucose after heating for one hour decreased with increased temperatures up to 26.7 C (Figure 2). Heat treatment at 26.7 C for one hour was sufficient to inactivate the enzymatic pathway necessary for oxygen uptake.

A rapid decrease in the rate of endogenous activity reflected by uptake of oxygen occurred at temperatures above 20 C (Figure 3). MPN values (Figure 8) after heat treatment of cells indicated that the 19.7 C and 23.1 C curves represent 10^{10} viable cells; the 26.3 C curve, 10^{8} viable cells.

Increased times of exposure at 25.3 C decreased the reaction rate in the presence of glucose (Figure 4). However, after 60 minutes exposure, a lag was noted in oxygen uptake. Observations for the 60 minute sample extended beyond 85 minutes demonstrate slight

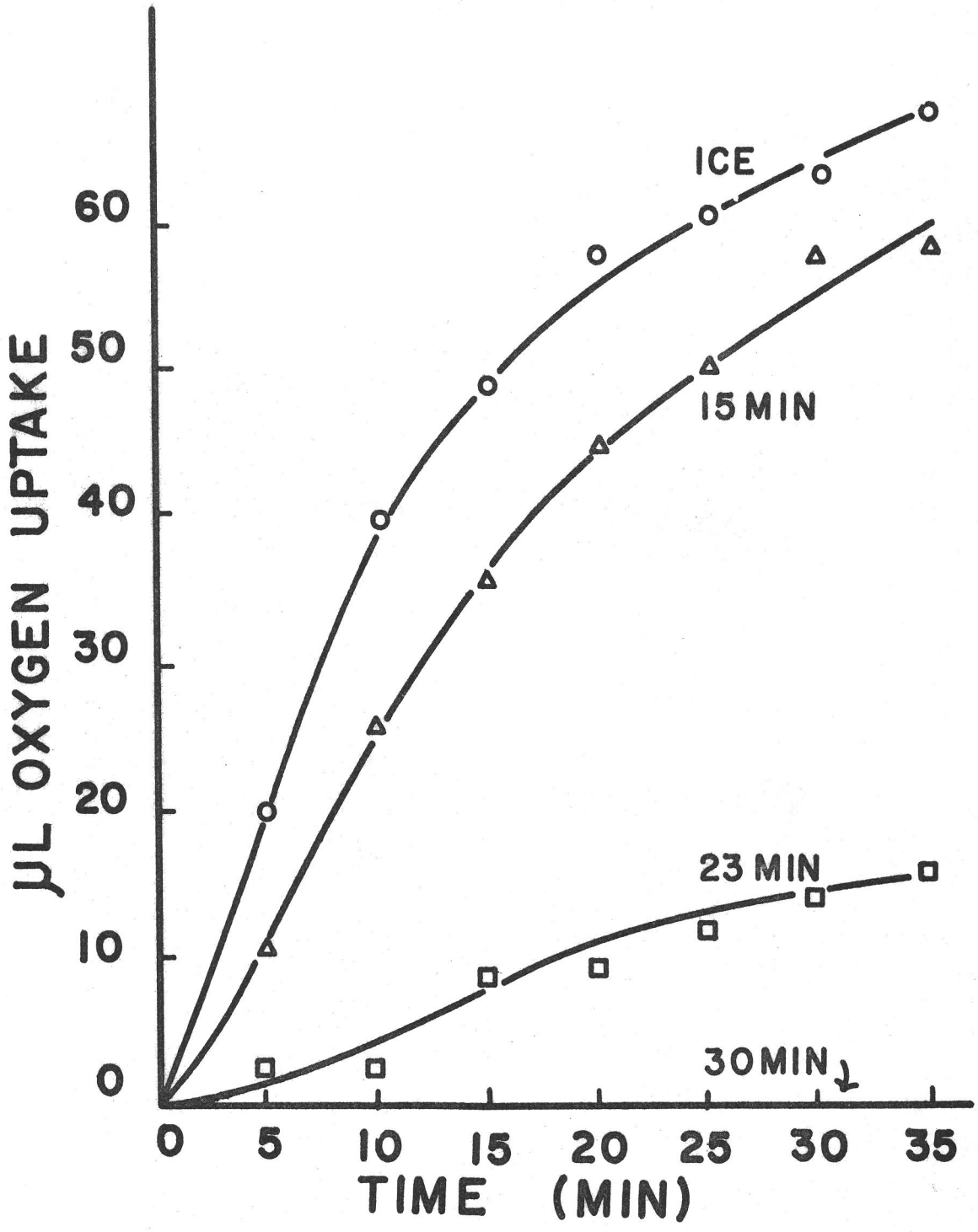

Figure 1. Oxygen uptake at 15.2 C by V. marinus MP-1 (1.41 mg N) in the presence of glucose (1.0 μmole) after heating at 29.4 C for designated times. Values corrected for endogenous controls.

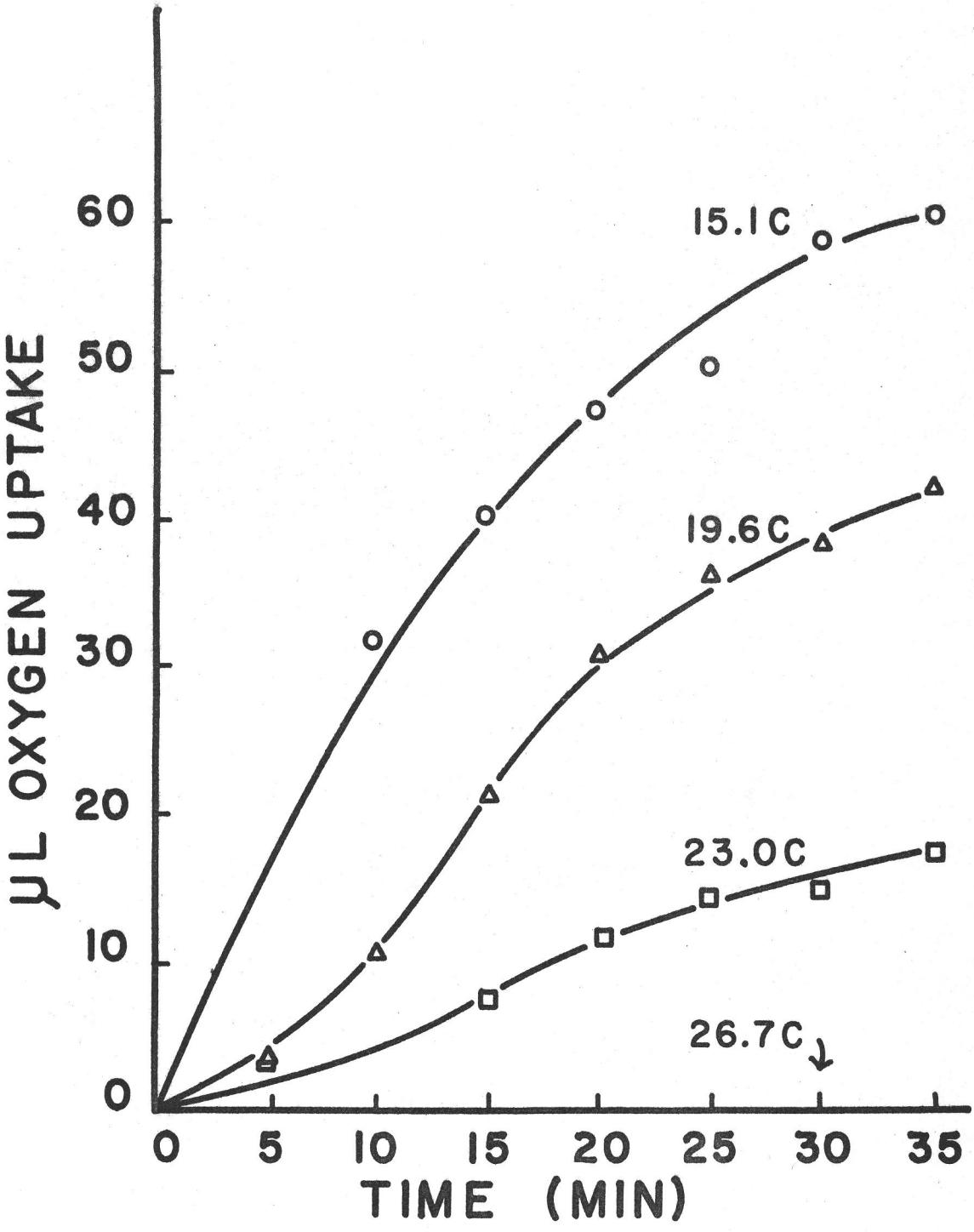

Figure 2. Oxygen uptake at 15. 2 C by V. marinus MP-1 (1. 41 mg N) in the presence of glucose (1. 0 μmole) after heating for one hour at temperatures indicated. Values corrected for endogenous controls.

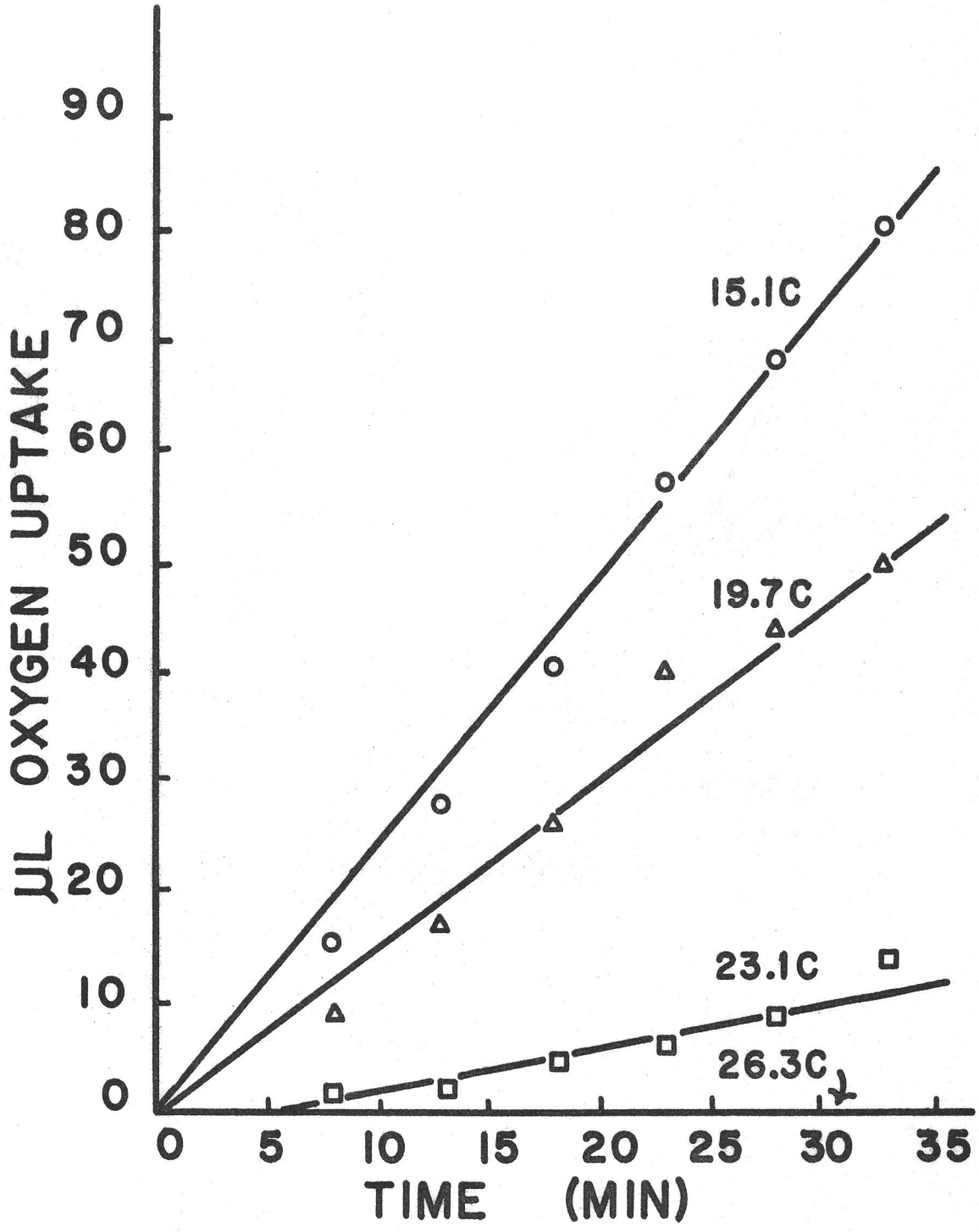

Figure 3. Endogenous oxygen uptake at 15.2 C by V. marinus MP-1 (1.50 mg N) after heating for one hour at temperatures indicated.

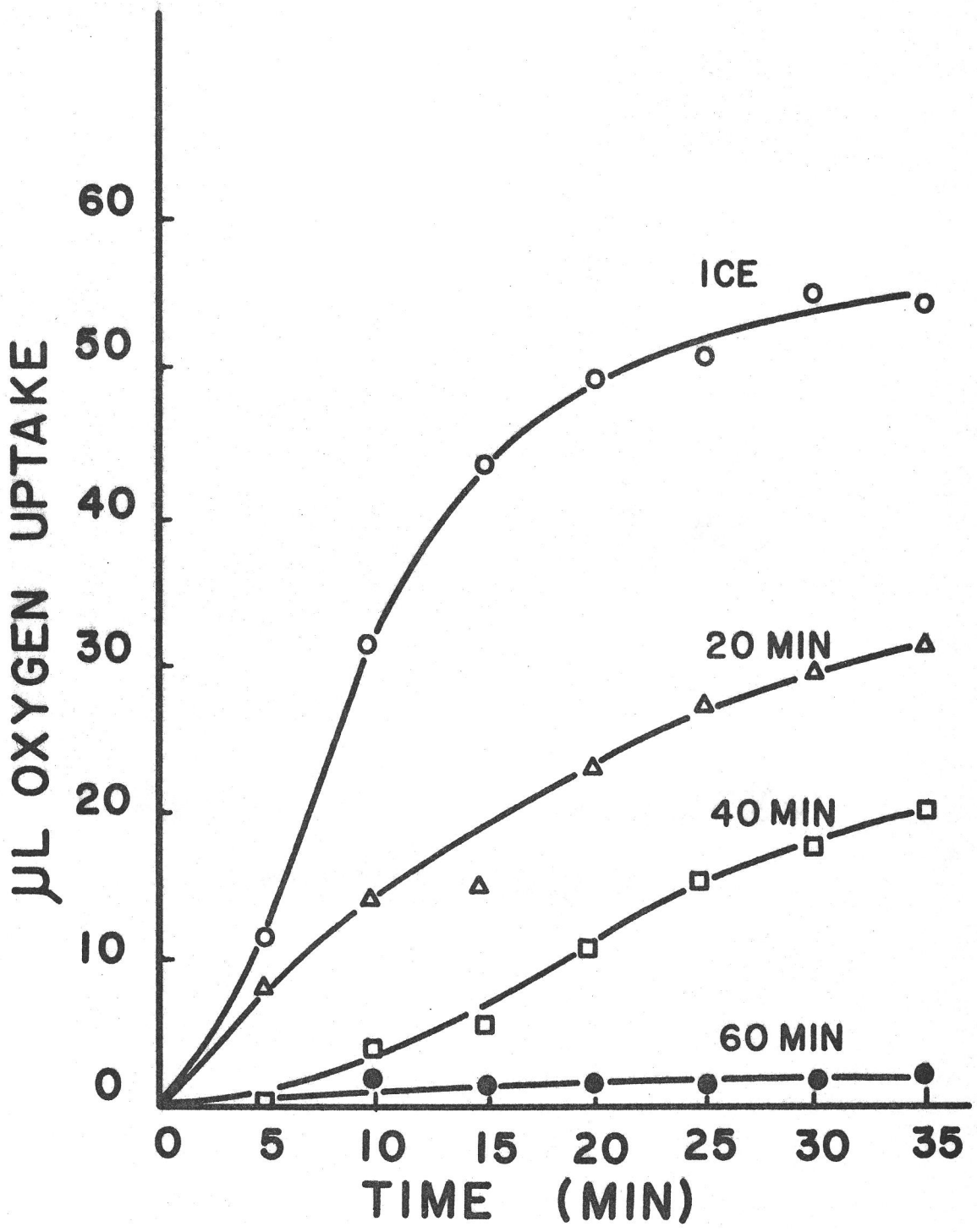

Figure 4. Oxygen uptake at 15.1 C by V. marinus MP-1 (1.36 mg N) in the presence of glucose (1.0 μmole) after heating at 25.3 C for designated times. Values corrected for endogenous controls.

oxygen uptake.

Figure 5 shows that endogenous activity also decreased. After 0 and 20 minutes exposure at 25.3 C there are 10^{10} viable cells. The 80 minute curve represents 10^{7} viable cells.

Cells heated at 20.8 C, approximately one degree above the maximum growth temperature, for prolonged periods showed a decrease in oxygen uptake in the presence of glucose (Figure 6). Endogenous activity, represented in Figure 7, was still present after 110 minutes. The number of viable cells decreased from 10^{10} to 10^{9} after 110 minutes.

Viability and Turbidity Studies

The number of viable cells remaining after heat exposure, dilution into and equilibration of Warburg flasks was estimated by MPN at designated intervals (Figure 8). The net drops corresponded to plate counts obtained from non-aerated temperature exposed cell suspensions before addition to Warburg flasks.

Heated cell suspensions decreased in turbidity at 600 mμ after an initial increase (Figure 9). The period of increase and rate of decrease depended on the time and temperature of exposure. A pronounced decrease occurred after 30 minutes at 29.2 C, after 40 minutes at 24.7 C and after 80 minutes at 20.4 C.

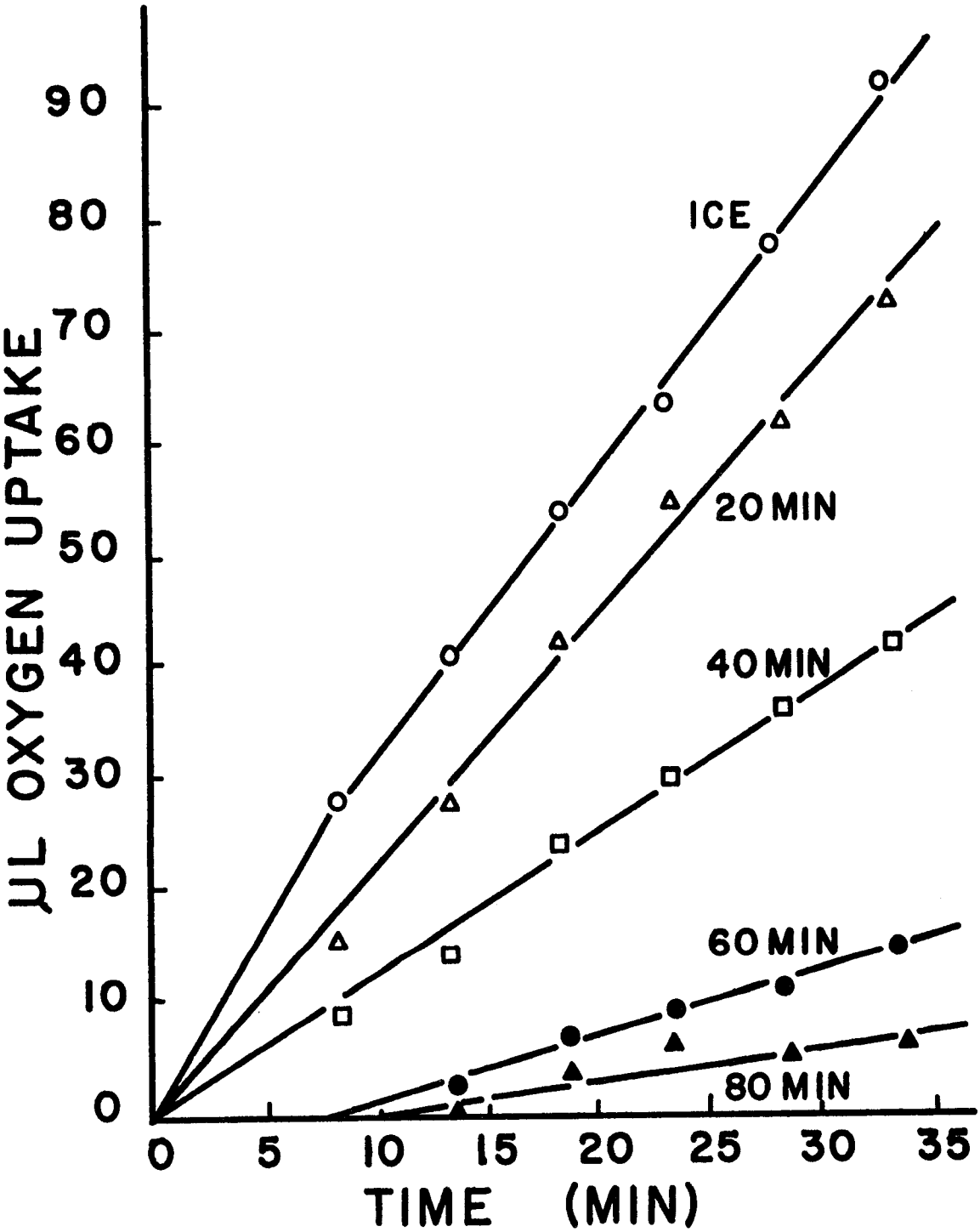

Figure 5. Endogenous oxygen uptake at 15.1 C by V. marinus MP-1 (1.36 mg N) after heating at 25.3 C for designated times.

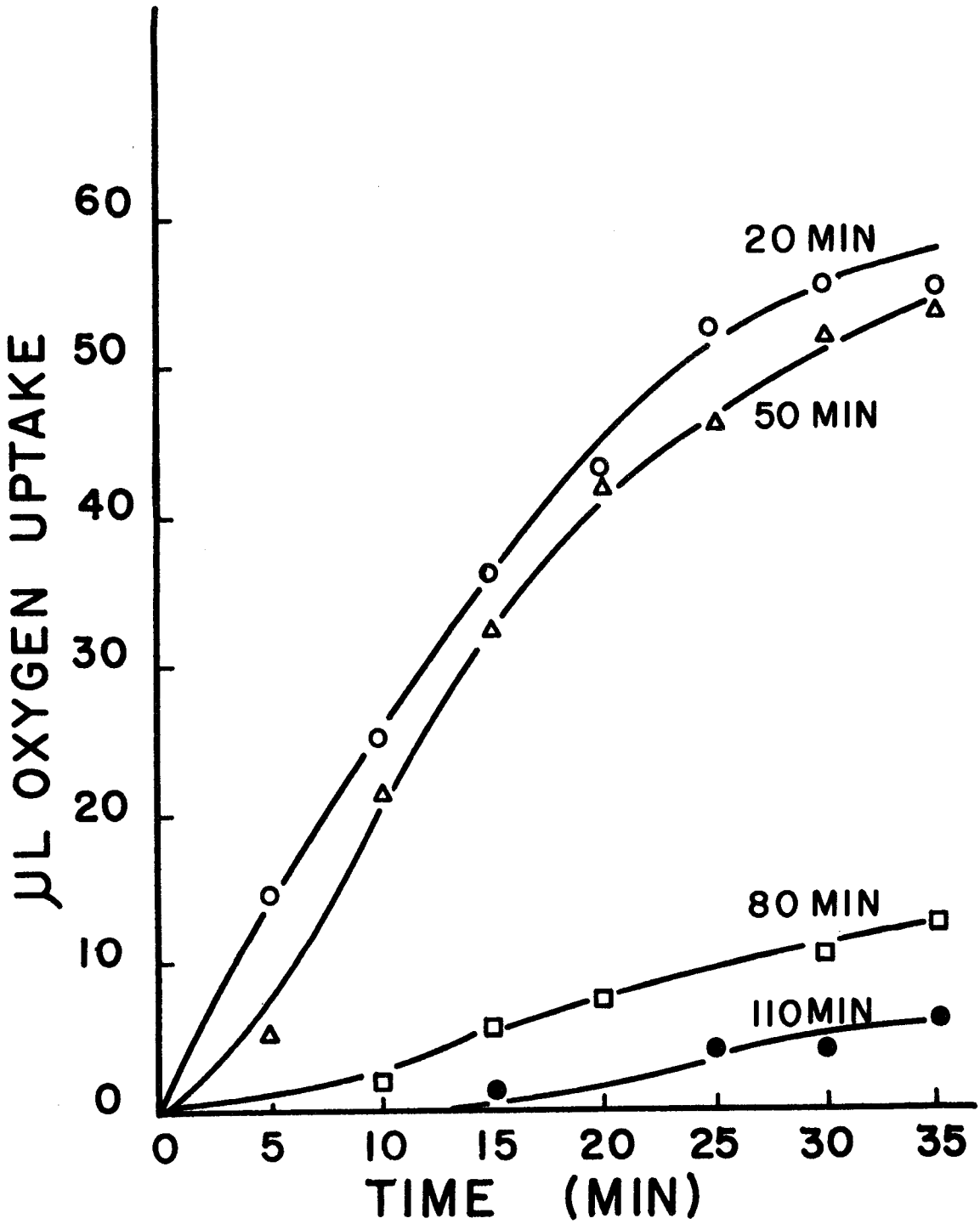

Figure 6. Oxygen uptake at 15.1 C by V. marinus MP-1 (1.38 mg N) in the presence of glucose (1.0 μmole) after heating at 20.4 C for designated times. Values corrected for endogenous controls.

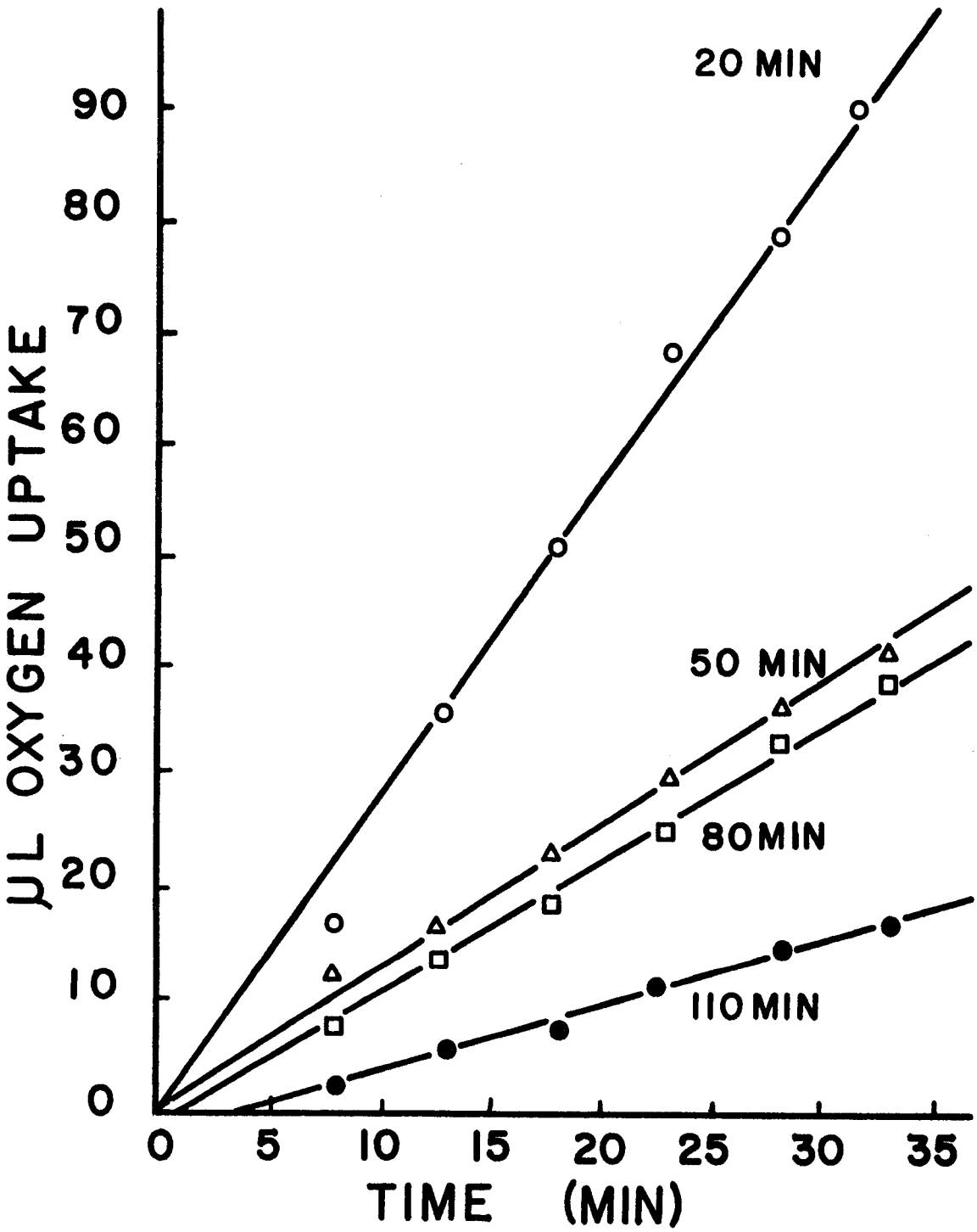

Figure 7. Endogenous oxygen uptake at 15.1 by V. marinus MP-1 (1.38 mg N) after heating at 20.4 C for designated times.

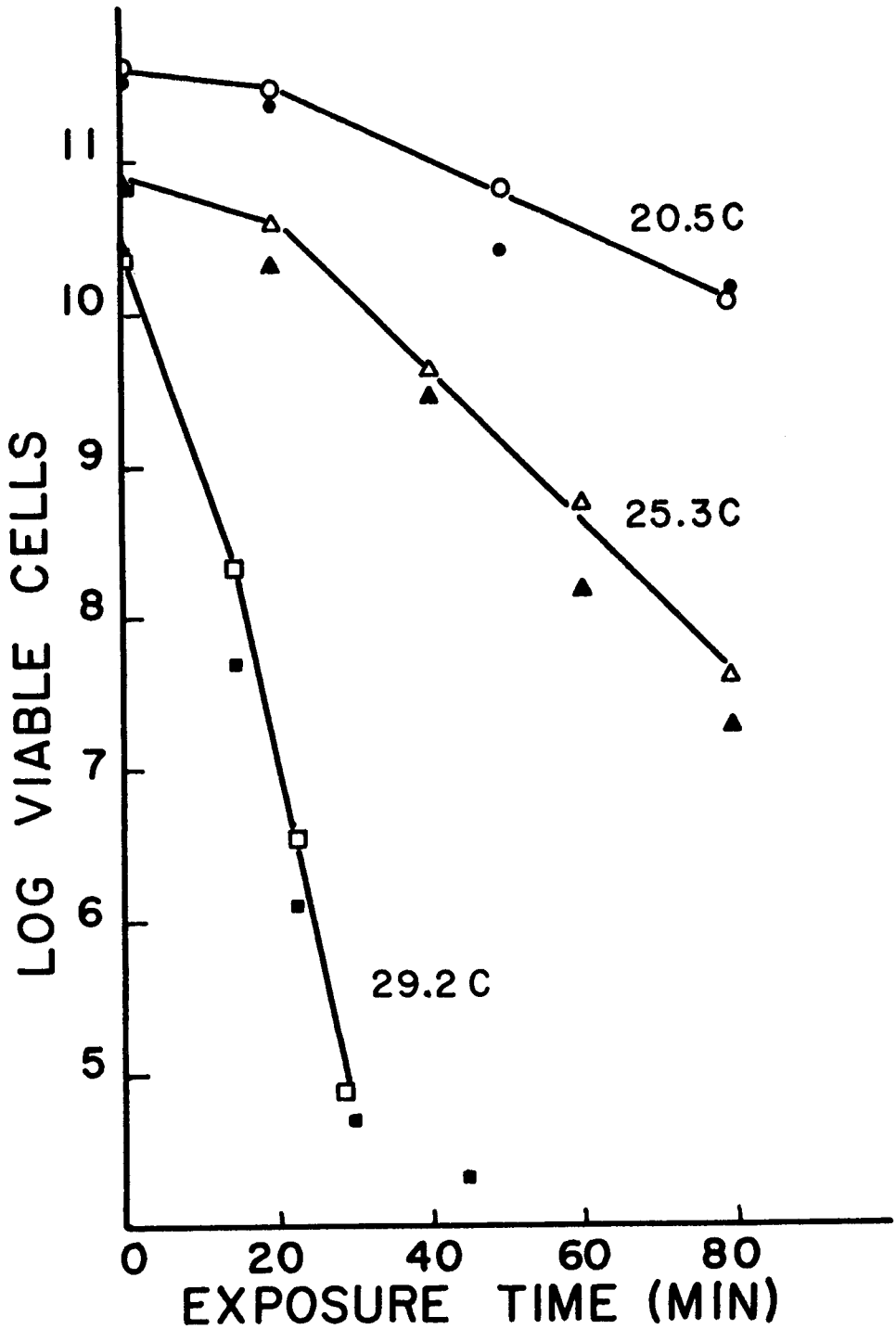

Figure 8. Viability studies without substrate after heating at designated temperatures for various times and equilibration for Warburg studies. Open figures represent zero time (first manometer readings); closed figures, 45 minutes (35 minutes for 20.5 C).

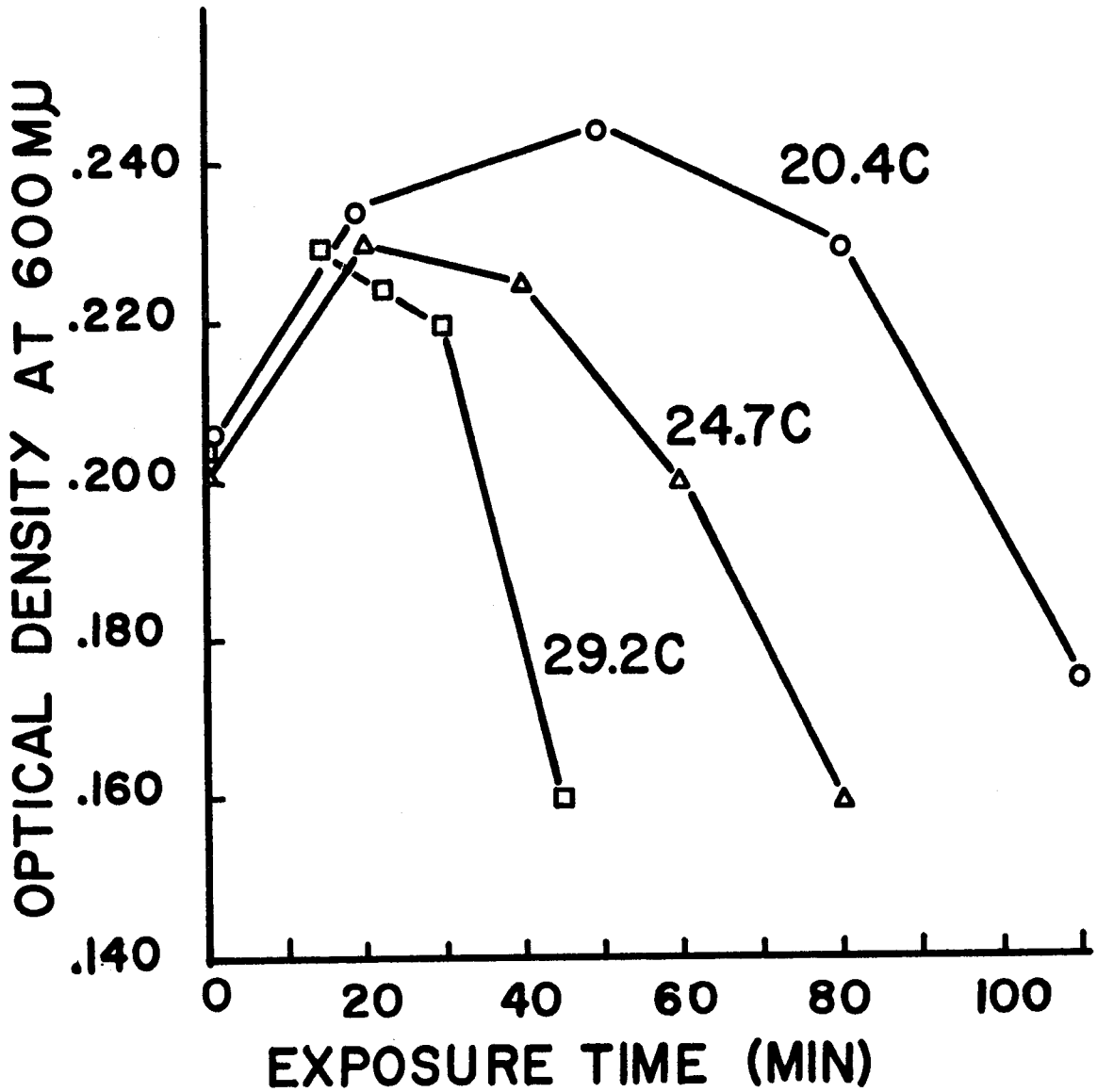

Figure 9. Changes in OD with time of whole cell suspensions of V. marinus MP-1 diluted 1:100 after heating at temperatures indicated.

Analysis of Supernatant

Ultraviolet absorbing materials were present in the supernatants obtained from heat treated cell suspensions of V. marinus MP-1. The absorption peak was approximately 260 mμ. Absorption at 260 mμ and 280 mμ increased with increased temperature (Table I) as well as increased exposure. The OD was lowest at 260 mμ and 280 mμ for suspensions heated for one hour at 15 C. Pronounced increases occurred above 20 C. Radical increases occurred above 28 C.

The supernatants were analyzed for Kjeldahl nitrogen. Leakage of nitrogen containing materials showed a similar pattern (Table I). A 25 percent increase occurred between 14. 8 C and 19. 2 C; a 35 percent increase between 19. 2 and 26. 0 C. Between 28. 5 C and 30. 5 C a 400 percent increase was noted. Between 33. 0 C and 35. 2 C the nitrogen content of the supernatant decreased.

When the ratio of 260 mμ to 280 mμ absorbing material was calculated by the method of Warburg and Christian (69) nucleic acid and protein also increased with increasing temperatures above the growth temperature of the organism (Table I). Slight differences are noted between these results, those obtained from Kjeldahl nitrogen and direct analysis on the supernatant for orcinol and diphenylamine reacting materials (Table I).

Orcinol reacting material, indicative of RNA, showed an even

TABLE I. PARTIAL ANALYSIS OF LEAKAGE MATERIAL IN SUPERNATANT FLUID FROM *V. MARINUS* MP-1 AFTER ONE HOUR.

Temp. °C	Kjeldahl Nitrogen µg/ml	Optical Density 260 mµ	Optical Density 280 mµ	260-280 mµ Protein µg/ml	260-280 mµ Nucleic Acid µg/ml	Orcinol Reacting Materials µg RNA/ml	Diphenylamine Reacting Materials µg DNA/ml
10.0	lost	0.880	0.620	316	33	22	-
14.8	144	0.630	0.385	146	24	19.5	-
19.2	175	0.745	0.500	240	29	19.5	-
26.0	231	1.163	0.835	420	43	31	-
28.5	288	1.449	1.012	470	93	60	±
30.5	1184	0.765*	0.452*	1630	326	405	23.0
33.0	1296	1.217*	0.660*		530	640	67.0
35.2	1254	1.351*	0.670		610	730	75.5

*1: 10 dilution of supernatant

more radical increase between 28. 5 and 30. 5 C than did Kjeldahl nitrogen. Diphenylamine reacting materials, indicative of DNA, were observed in the supernatant above 28. 5 C.

Supernatants from cell suspensions heated above 29 C were yellow. Spectrophotometric analysis showed a distinct absorption peak at 418 mμ. Figure 10 represents the leakage spectra between 395 and 435 mμ for supernatants obtained from cell suspensions heated for one hour at 24. 7 C, 29. 2 C, 33. 7 C and 38. 0 C.

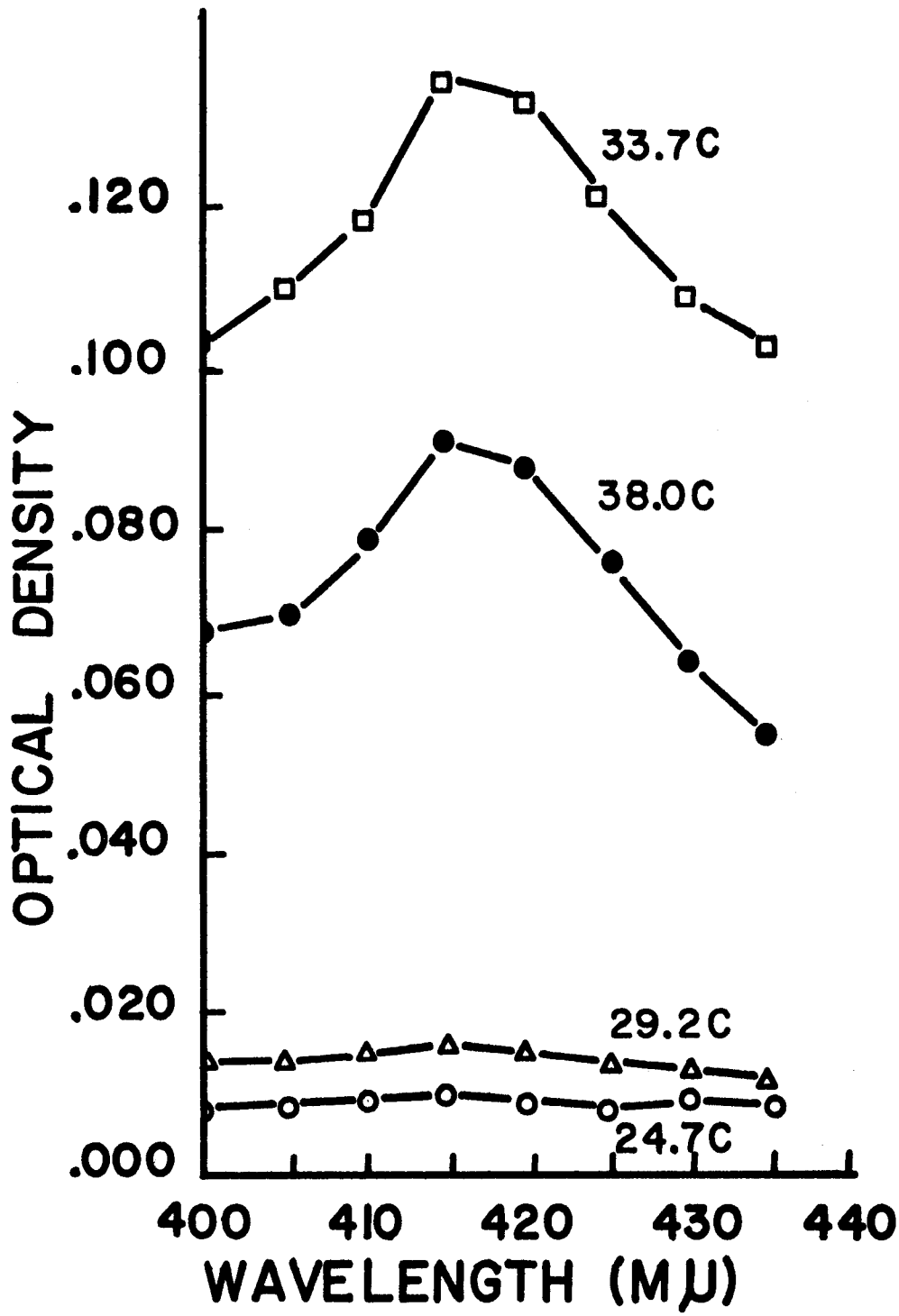

Figure 10. Thermal leakage spectra (400-435 mμ) of V. marinus MP-1 after heating for one hour at the temperatures indicated.

DISCUSSION

Rapid growth of bacteria at temperatures near 0 C has been observed. These psychrophilic bacteria carry out all the significant physiological activities at temperatures which ordinarily inhibit other microorganisms. It should be remembered that no obligate psychrophilic bacterium has been described as to its taxonomy, physiology, biochemical characteristics and temperature requirements other than V. marinus (11, 15, 41, 42).

Edwards and Rettger (20) have suggested that interference with mechanisms involved in respiration would result either in loss of ability to respire and to remain viable or in substitution of a different respiratory system.

Observations showed that after exposure to 29. 4 C there was a rapid decrease in oxygen uptake and viability with increased time. After 30 minutes exposure oxygen uptake was negligible (Figure 1). The data indicate that only 0. 0001 percent of the cells retained the ability to repair cellular damage after exposure to 29. 4 C for 43 minutes. At 29. 4 C the thermolability of V. marinus MP-1 cells is expressed very rapidly resulting in the loss of the ability to take up oxygen in the presence of glucose.

The temperature response is well noted at temperatures above the organisms maximum growth temperature (Figure 2). MPN values

clearly demonstrate that viable cells are still present at 25. 3 C for one hour (Figure 8), although only negligible oxygen uptake can be demonstrated. The data from Figure 2 and Figure 3 show that 19. 7 C and 23. 1 C also affect metabolic factors involved in oxygen uptake.

Prolonged lag periods in oxygen uptake after exposure to 20. 4 C and 25. 3 C could indicate that the organisms repaired cellular damage due to heat. The prolonged lag in the presence of glucose after 60 minutes exposure at 25. 3 C shows that the mechanism for oxidation of glucose was inactivated before that for endogenous oxygen consumption. This could also be the result of an increase in substrate induced death after heat exposure. Postgate and Hunter (55) reviewed substrate induced death.

Endogenous activity after exposure to 20. 4 C for intervals ranging from 45 to 80 minutes decreases slightly. Apparently during this time there was little effect of temperature on the mechanisms involved in the oxidation of endogenous reserves accompanied by an approximately five fold decrease in viability.

These manometric studies indicate the presence of some enzyme(s) of a psychrophilic nature within the organism and the low energy of thermal inactivation of the enzyme(s). Whether it is the thermal inactivation by moderate temperature of one enzyme in the metabolic pathway from glucose utilization to the production of carbon dioxide or of several enzymes still remains to be solved. It is also possible that all the enzymes involved in the uptake of oxygen for glucose metabolism are adversely affected by moderate

temperature. The same can be said for those enzymes involved with endogenous oxygen uptake. A time-temperature relation is definitely indicated in the thermolability of the enzymes involved in oxygen uptake endogenously or with glucose as a substrate. This study is a basis for a search for psychrophilic enzymes.

Even though oxygen uptake may be destroyed, some of the cells still have the ability to remain viable, to repair damage and to reproduce as expressed in MPN values. This may be at the expense of the cells rendered nonviable or due to some more resistant cells in the population.

Postgate and Hunter (55) reviewed the influence of population density on death and death time curves. One inoculum size is not valid for another. They did not find MPN studies sensitive enough for most of their studies, although they did use them to determine the best recovery medium. Nelson (43, 44) found that dilution counts in liquid media were superior to plate counts with similar media. MPN studies of V. marinus MP-1 exposed to moderate temperatures compared favorably with spread plate observations using a different media. Both methods have inherent sources of error and are affected by cell clumping. MPN results were relatively more uniform and were employed in these studies.

Hagen, Kushner and Gibbons (23) recognized a 90 percent drop in viability with their obligate psychrophile before a drop in

turbidity occurred. Slight decreases in turbidity of cell suspensions of MP-1 occur before 90 percent of the cells are rendered nonviable at all temperatures tested with the possible exception of those above 28 C. However, pronounced decreases in turbidity did not occur until over 90 percent of the cell population was no longer viable. This indicates that lysis is not the initial step in heat inactivation of the viability mechanisms.

If heat can alter the cell membrane so that it can no longer control what enters and leaves the cell, one would expect to observe temporary to permanent loss in selective permeability to certain cellular constituents. Slight increases in temperature above the maximum for growth might produce temporary or repairable damage, while slightly higher temperatures could produce permanent damage.

Changes in the turbidity of the cell suspensions of MP-1 following temperature exposure indicated a decrease in cell size due to utilization of or leakage of cellular material or possible lysis. This would in turn alter the composition of the supernatant.

Postgate and Hunter (54) found that the osmotic barrier temporarily remains intact after starvation induced death as indicated by impermeability to anilino-napthalene sulphonic acid type dyes. Ecker and Schaecter (19) noted a decrease in cell size under conditions of limited nutrition. Newton (45) observed a decrease in electron dense material corresponding to losses in nucleic acid content.

Preliminary examination of the supernatant from MP-1 revealed an absorption peak at about 260 mμ. This implicates the presence of nucleic acid or degradation products of nucleic acids. Leakage of 260 mμ absorbing materials is not a sufficient criterion for lysis. The release of these compounds from Bacillus cereus (68), Escherichia coli (29, 59), Aerobacter aerogenes (61, 62), Saccharomyces cereviseae (27) and Pseudomonas aeruginosa (22) was not due to cell lysis.

Gronlund and Campbell (22) observed the release of ultraviolet absorbing materials from Ps. aeruginosa with time and with no concomitant loss of viability. This consisted of RNA material. No DNA material was detected in the supernatant. Endogenous utilization of RNA has been demonstrated in Ps. aeruginosa (12, 21, 22), A. aerogenes (6, 54, 55, 61, 63), E. coli (17, 18), Sarcina lutea (9) and Lactobacillus arabinosa (28).

The data presented in Table I for leakage of nitrogenous materials and nucleic acids suggest that this may also be true with cell suspensions of V. marinus MP-1 heated for one hour at temperatures up to 28.5 C. However, the radical increases incurred above 28.5 C indicate that the leakage is no longer a selective process.

Herbst and Doctor (25) have studied the effect of temperature and pH with and without spermine on the leakage of Haemophilus parainfluenzae suspended in tris buffer. Leakage was most

pronounced at 37 C (optimal temperature for growth of the organism), whereas little or no loss occurred at low temperatures. The plot of 260 mμ absorbing material leaked out vs temperature resembles that of V. marinus MP-1, except that the peak in the latter case is between 34 and 35 C, well above the physiological range of the organism. The decrease in the material from MP-1 appearing in the supernatant at the higher temperatures probably resulted from clumping of cells and coagulation trapping quantities of material or from utilization of breakdown products.

Strange and Schon (62) found that A. aerogenes grown in defined medium degraded RNA at an almost linear rate at temperatures between 30 and 47 C. The death rate below 44 C was negligible. Depletion of RNA at 47 C was not sufficient to account for the lethal effect.

More specific analysis of MP-1 supernatants for orcinol reacting material (indicative of RNA) showed a similar pattern to that of Kjeldahl nitrogen. Diphenylamine reacting material appeared only at temperatures above 28.5 C for one hour. DNA is generally regarded as stable and may increase in the undividing cell during starvation (61). Harrison and Lawrence (22) reported the disappearance of DNA after 19 hours in A. aerogenes by analysis of cell material and not of supernatant. Therefore, the appearance of DNA material in the supernatant would strongly implicate lysis. Leakage of

materials having a maximum absorbtion peak at 418 mμ (Figure 10) and 553 mμ were also present. Leakage of DNA materials and materials absorbing in the cytochrome range strongly implicates lysis or loss of selective permeability after one hour at temperatures above 28 C.

Several workers have noted the effect of growth phase and growth rate on death of endogenously respiring bacteria (54, 56, 61, 62, 70). In general, permeability effects and heat lability are greatest during the logarithmic phase and less during the stationary phase. Thus, one would expect any effect of temperature to manifest itself most vividly at this stage.

The order of changes observed with increased temperature on cell suspensions of MP-1 were as follows: 1) protein and RNA material leak slowly from cells, 2) slight increase in OD of the cell suspension occurs during which 3) the number of viable cells begins to decrease, 4) OD of the cell suspension decreases slightly followed by a rapid decrease, 5) the ability to consume oxygen in the presence of glucose is destroyed, 6) the endogenous oxygen consumption becomes negligible, 7) radical increases occur in RNA material present in the supernatant and 8) DNA material appears in the supernatant.

On the basis of the foregoing data, V. marinus MP-1 possesses an obligate psychrophilic nature. The sequence of events revealed

in these experiments with increased moderate temperatures became apparent only after considerable losses in viability had occurred. However, they are reflections of the processes which rendered the cells nonviable. Therefore, the enzymes necessary for the reactions involved in the replacement of damaged cell materials and glucose oxidation must have been inactivated by moderate temperatures.

These studies lay the foundation for further research on the basic reasons for biochemical lesions, loss of permeability and enzyme inactivation produced by moderate temperature on an obligately psycrophilic organism.

SUMMARY

Moderate temperatures of 20 to 30 C destroy the mechanisms essential for V. marinus MP-1 to consume oxygen endogenously and in the presence of glucose. Even though oxygen uptake may be destroyed by heat, some of the cells still have the ability to remain viable, to repair damage and to reproduce as expressed in MPN values.

The effect of higher temperatures and longer exposure periods can be partially explained in terms of thermal action to produce cellular damage and biochemical lesions to bring about the expiration of cells.

Leakage of nitrogenous materials indicates a pronounced effect on cellular composition and permeability.

Twenty-eight C for 45 minutes is sufficient to produce radical changes in the optical density of a cell suspension and the amount of orcinol reacting material found in the supernatant. Diphenylamine reacting material appeared and oxygen uptake was destroyed under these conditions.

The preceeding data helps verify the obligate psychrophilic nature of V. marinus MP-1.

BIBLIOGRAPHY

1. American Public Health Association, American Water Works Association, and Water Pollution Control Federation. Standard methods for the examination of water and waste water including bottom sediments and sludges. 11th ed. New York, 1960. 625 p.

2. Azuma, Y. and L. D. Witter. Osmotic fragility of protoplasts of a mesophilic and a psychrophilic Pseudomonas aeruginosa. In: Bacteriological Proceedings, Abstracts of the 64th Annual Meeting, Washington, D. C. Ann Arbor, Michigan, American Society for Microbiology, 1964. p. 33.

3. Baxter, R. M. and N. E. Gibbons. Observations on the physiology of psychrophilism in a yeast. Canadian Journal of Microbiology 8:511-517. 1962.

4. Bedford, R. H. Marine bacteria of the northern Pacific Ocean. The temperature range for growth. Contributions to Canadian Biology and Fisheries, ser. C 8:461-474. 1933.

5. Berry, J. A. and A. Magoon. Growth of microorganisms at and below 0° C. Phytophathology 24:780-796. 1934.

6. Borek, Ernst, A. Ryan and J. Rockenbach. Nucleic acid metabolism in relation to the lysogenic phenomenon. Journal of Bacteriology 69:460-467. 1955.

7. Borek, Ernst and Heinrich Waelsch. The effect of temperature on the nutritional requirement of microorganisms. Journal of Biological Chemistry 190:191-196. 1951.

8. Brown, A. D. Some general properties of a psychrophilic pseudomonad: Effects of temperature on some of these properties and utilization of glucose by this organism and Pseudomonas aeruginosa. Journal of General Microbiology 17:640-648. 1957.

9. Burleigh, I. G., E. A. Dawes, and D. W. Ribbons. Endogenous metabolism and survival of Sarcina lutea. The Biochemical Journal 88:30-31. 1963.

10. Burton, K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. The Biochemical Journal 62:315-322. 1956.

11. Burton, Sheril D. and Richard Y. Morita. Denaturation and renaturation of malic dehydrogenase in a cell-free extract from a marine psychrophile. Journal of Bacteriology 86:1019-1024. 1963.

12. Campbell, J. J. R., Audrey F. Gronlund, and Margaret G. Duncan. Endogenous metabolism of Pseudomonas. Annals of the New York Academy of Sciences 102:669-677. 1963.

13. Campbell Soup Company. Proceedings: Low Temperature Microbiology Symposium, 1961. Camden, N. J. 1962. 322 p.

14. Cirillo, Vincent P., Peter O. Wilkins, and Joseph Anton. Sugar transport in a psychrophilic yeast. Journal of Bacteriology 86:1259-1265. 1963.

15. Colwell, Rita R. and Richard Y. Morita. Reisolation and emendation of description of Vibrio marinus (Russell) Ford. Journal of Bacteriology 88:831-837. 1964.

16. Dawes, E. A. and D. W. Ribbons. The endogenous metabolism of microorganisms. Annual Reviews of Microbiology 16:241-264. 1962.

17. _____ Effect of environmental conditions on the endogenous metabolism of Escherichia coli. The Biochemical Journal 84:97P-98P. 1962.

18. _____ Endogenous metabolism and survival of Escherichia coli. Journal of Applied Bacteriology 26:vi. 1963.

19. Ecker, R. E. and M. Schaecter. Bacterial growth under conditions of limited nutrition. Annals of the New York Academy of Sciences 102:549-563. 1963.

20. Edwards, O. F. and L. F. Rettger. The relation of certain respiratory enzymes to the maximum growth temperature of bacteria. Journal of Bacteriology 34:489-515. 1937.

21. Gronlund, Audrey F. and J. J. R. Campbell. Nitrogenous compounds as substrates for endogenous respiration in microorganisms. Journal of Bacteriology 81:721-724. 1961.

22. _____ Nitrogenous substrates of endogenous respiration in Pseudomonas aeruginosa. Journal of Bacteriology 86: 58-65. 1963.

23. Hagen, P. O., D. J. Kushner and N. E. Gibbons. Temperature-induced death and lysis in a psychrophilic bacterium. In: Bacteriological Proceedings, Abstracts of the 64th Annual Meeting, Washington, D. C. Ann Arbor, Michigan, American Society for Microbiology, 1964. p. 42.

24. Harrison, A. P. Jr. and F. R. Lawrence. Phenotypic, genotypic and chemical changes in starving populations of Aerobacter aerogenes. Journal of Bacteriology 85:742-750. 1963.

25. Herbst, Edward J. and B. P. Doctor. Inhibition of ribonucleic acid degradation by spermine. Journal of Biological Chemistry 234:1497-1500. 1959.

26. Hess, E. Effect of low temperature on the growth of marine bacteria. Contributions to Canadian Biology and Fisheries, ser. C 8:491-505. 1934.

27. Higuichi, M. and T. Uemura. Release of nucleotides from yeast cells. Nature 184:1381-1383. 1959.

28. Holden, J. T. Degradation of intracellular nucleic acids and leakage of fragments by Lactobacillus arabinosus. Biochimica et Biophysica Acta 29:667-668. 1958.

29. Horiuchi, Tadao. RNA degradation and DNA and protein synthesis of E. coli B. in a phosphate deficient medium. Journal of Biochemistry 46:1467-1480. 1959.

30. Ingraham, J. L. and G. R. Bailey. Comparative study of the effect of temperature on metabolism of psychrophilic bacteria. Journal of Bacteriology 77:609-613. 1959.

31. Ingraham, J. L. and J. L. Stokes. Psychrophilic bacteria. Bacteriological Reviews 23:97-108. 1959.

32. Kates, M. and R. M. Baxter. Lipid composition of mesophilic and psychrophilic yeasts (Candida species) as influenced by environmental temperature. Canadian Journal of Biochemistry and Physiology 40:1213-1227. 1962.

33. Kates, M. and P. O. Hagen. Influence of temperature on fatty acid composition of psychrophilic and mesophilic Serratia marcescens. Canadian Journal of Biochemistry 42:481-488. 1964.

34. Lamana, Carl. Studies on endogenous metabolism in bacteriology. Annals of the New York Academy of Sciences 102:517-520. 1963.

35. Lewis, Roger W. Temperature and pressure effects on the fatty acids of some marine ectotherms. Comparative Biochemistry and Physiology 6:75-89. 1962.

36. Lichstein, Herman C. and William J. Begue. Increased nutritional requirements of Saccharomyces cereviseae as a result of incubation at 38 C. Proceedings of the Society for Experimental Biology and Medicine 105:500-504. 1960.

37. Lindeberg, Goesta and Aaslang Lode. Release of ultraviolet-absorbing material from Escherichia coli at subzero temperatures. Canadian Journal of Microbiology 9:523-530. 1963.

38. Mallette, M. Frank. Validity of the concept of energy of maintenance. Annals of the New York Academy of Sciences 102:521-535. 1963.

39. Mandelstam, J. The intracellular turnover of protein and nucleic acids and its role in biochemical differentiation. Bacteriological Reviews 24:289-308. 1960.

40. Marr, Allen G. and John L. Ingraham. Effect of temperature on the composition of fatty acids in Escherichia coli. Journal of Bacteriology 84:1260-1267. 1962.

41. Morita, Richard Y. and Sheril D. Burton. Influence of moderate temperature on growth and malic dehydrogenase activity of a marine psychrophile. Journal of Bacteriology 86:1025-1029. 1963.

42. Morita, Richard Y. and Roger D. Haight. Temperature effects on the growth of an obligate psychrophilic marine bacterium. Limnology and Oceanography 9:103-106. 1964.

43. Nelson, F. E. Factors which influence the growth of heat treated bacteria. Journal of Bacteriology 45:395-403. 1934.

44. Nelson, F. E. Factors which influence the growth of heat treated bacteria. Journal of Bacteriology 48:473-477. 1944.

45. Newton, B. A. The release of soluble constituents from washed cells of Pseudomonas aeruginosa by action of polymyxin. Journal of General Microbiology 9:54-64. 1953.

46. Niwa, M., Y. Yamadeya and Y. Kuwajima. Leakage of cell components of Bordetella pertusis. Journal of Bacteriology 88:809-810. 1964.

47. Okabayashi, Tadashi et al. Occurrence of nucleotides in the culture fluids of microorganisms IV. On the character of Brevibacterium liquefaciens novo sp. and its cultural conditions for excretion of nucleotides. Annual Report of Shionogi Research Laboratory 12:191-197. 1962.

48. Okabayashi, Tadashi, Misao Ide and Akihiro Yoshimoto. Excretion of adenosine-3', 5' phosphate in the culture broth of Brevibacterium liquefaciens. (Letters to the editors) Archives of Biochemistry and Biophysics 100:158-159. 1963.

49. Okabayashi, Tadashi and Eitaro Masuo. Occurrence of nucleotides in the culture fluid of a microorganism. Chemical and Pharmaceutical Bulletin 8:370-372. 1960.

50. _____ Occurrence of nucleotides in the culture fluid of microorganisms I. Screening of purine excreting bacteria with purine auxotrophs of Escherichia coli. Chemical and Pharmaceutical Bulletin 8:1084-1088. 1960.

51. _____ Occurrence of nucleotides in the culture fluid of microorganisms II. The nucleotides in the broth of Brevibacterium liquefaciens novo sp. Chemical and Pharmaceutical Bulletin 8:1089-1094. 1960.

52. Okabayashi, Tadashi, Akihiro Yoshimoto and Misao Ide. Occurrence of nucleotides in culture fluids of microorganisms V. Excretion of adenosine cyclic 3', 5'-phosphate by Brevibacterium liquefaciens sp. N. Journal of Bacteriology 86:930-936. 1963.

53. Oppenheimer, C. H. and W. Drost-Hansen. A relationship between multiple optima for biological systems and the properties of water. Journal of Bacteriology 80:21-29. 1960.

54. Postgate, J. R. and J. R. Hunter. Survival of starved bacteria. Journal of General Microbiology 29:233-263. 1962.

55. _____ The survival of starved bacteria. Journal of Applied Bacteriology 26:295-306. 1963.

56. Ribbons, Douglas W. and Edwin A. Dawes. Environmental and growth conditions affecting the endogenous metabolism of bacteria. Annals of the New York Academy of Sciences 102: 564-586. 1963.

57. Rose, Anthony H. Temperature relationships among microorganisms. Wallerstein Communications 25:5-16. 1962.

58. Schneider, W. C. Determination of nucleic acids in tissues by pentose analysis. In: Methods in enzymology, ed. S. P. Colowick and N. P. Kaplan. Vol. 3. New York, Academic Press, 1957. p. 680-684.

59. Stephenson, M. and J. M. Moyle. Nucleic acid metabolism of Escherichia coli. The Biochemical Journal 45:vii. 1949.

60. Stokes, J. L. General biology and nomenclature of psychrophilic bacteria. In: Recent progress in microbiology: Symposia held at the VIII International Congress for Microbiology, Montreal, ed. N. E. Gibbons. Toronto, University of Toronto Press, 1963. p. 187-192.

61. Strange, R. E., F. A. Dark and A. G. Ness. The survival of stationary phase Aerobacter aerogenes stored in aqueous suspension. Journal of General Microbiology 25:61-76. 1961.

62. Strange, R. E and M. Schon. Effects of thermal stress on viability and ribonucleic acid of Aerobacter aerogenes in aqueous suspension. Journal of General Microbiology 34:99-114. 1964.

63. Strange, R. E., H. E. Wade, and A. G. Ness. The catabolism of protein and nucleic acids in starved Aerobacter aerogenes. The Biochemical Journal 86:197-203. 1963.

64. Sultzer, Barnet M. Oxidative activity of psychrophilic and mesophilic bacteria on saturated fatty acids. Journal of Bacteriology 82:492-497. 1961.

65. Takahashi, I. and N. E. Gibbons. Effect of salt concentration on the extracellular nucleic acids of Micrococcus halodenitrificans. Canadian Journal of Microbiology 3:687-694. 1957.

66. Upadhyay, J. and J. L. Stokes. Temperature sensitive formic hydrogenlyase in a psychrophilic bacterium. Journal of Bacteriology 85:177-185. 1963.

67. Upadhyay, J. and J. L. Stokes. Temperature-sensitive hydrogenase and hydrogenase synthesis in a psychrophilic bacterium. Journal of Bacteriology 86:992-998. 1963.

68. Urba, R. C. Protein breakdown in Bacillus cereus. The Biochemical Journal 71:513-518. 1959.

69. Warburg, O. and W. Christian. Isolierung und Kristallization des Gärungsferments Enolase. Biochemische Zeitschrift 310:384-421. 1942.

70. Winslow, C.-E. A. and Harold H. Walker. The earlier phases of bacterial culture cycle. Bacteriological Reviews 3:147-186. 1939.

71. ZoBell, C. E. Microbiological activities at low temperature with particular reference to marine bacteria. The Quarterly Review of Biology 9:460-466. 1934.

72. ZoBell, C. E. and J. E. Conn. Studies of the thermal sensitivity of marine bacteria. Journal of Bacteriology 40:223-238. 1940.