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Title GENERAL STUDIES OF TEMPERATURE EFFECTS ON AN OBLIGATE PSYCHROPHILE, VIBRIO MARINUS

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Vibrio marinus, strain MP-1, was shown to grow from pH 6.7 to 8.5, with an optimum at 7.3. Growth was inhibited below salinity 10 ‰ and above 60 ‰. The upper temperature limit of growth was 20 C, and growth was very rapid with aeration at 15 C.

Heating of the culture in Rila sea water at temperatures above 20 C caused severe viability loss which was inhibited by nutrient presence at 25 C. This loss was partially attributed to a thermally-induced membrane lesion.

The melting point (Tm) of DNA was 89.0 C; the percent extractable lipid content of the culture did not change with growth temperature.

A thermal lesion was shown to be a membrane rupture by analysis of the protein, RNA, DNA and amino acids which were released during heat shocking. Amino acid disproportionation occurred with increase in the time and temperature of heating, probably due to a

change in the hydrophobic-hydrophilic environment induced by heating. The RNA released was both polymeric and degraded. Heating caused the release of protein which was enzymatically active. A method for obtaining partially purified malic dehydrogenase from this culture was described.

GENERAL STUDIES OF TEMPERATURE EFFECTS ON AN OBLIGATE PSYCHROPHILE, VIBRIO MARINUS

by

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INTRODUCTION

Many organisms, generally unclassified, have been found in the marine environment (1, 29, 39, 40, 41). Few investigators have actively looked to the oceans as sources of psychrophiles. As 90 percent of the marine environment is at a temperature of 5.0 C or lower (39), the psychrophiles must comprise a major fraction of the bacteria which are active in biological cycles and mineralization processes. For a comprehensive assessment of in situ bacterial activity many factors must be considered; oxygen availability, salinity variation, substrate availability, pH, oxidation-reduction conditions, intrabacterial synergism, and temperature are all important factors. Any attempt to study all of these parameters at one time would be enlightening but extremely difficult. Consequently, most investigations of marine bacteria have been limited to general characterizations.

In the past psychrophilism has been studied primarily in connection with low temperature food spoilage. Other areas, not directly concerned with the applied aspects, have generally been neglected.

Obligate psychrophiles, as defined by Stokes (37), have only recently been shown conclusively to exist. Most studies to date on psychrophiles have been on facultatively psychrophilic cultures. Numerous

obligately psychrophilic bacteria have been isolated recently in this laboratory, a fact which should lead to more rigorous investigations of these widely spread bacteria.

Since an underlying knowledge of psychrophilism is basic to a real understanding of bacterial activity within the marine psychrosphere, this thesis will deal with pH, salinity, and temperature characteristics of the obligately psychrophilic marine bacterium, Vibrio marinus. This investigation, of a general and fundamental nature, serves as a basis for further and more detailed investigations of obligate psychrophilism, both physiologically and ecologically.

REVIEW OF LITERATURE

The recent isolation of an obligately psychrophilic marine bacterium (24) has afforded the opportunity to study obligate psychrophilism both microbiologically and biochemically. This strain, MP-1, was one of the series described by Colwell and Morita (4) as Vibrio marinus. Morita and Haight (24) reported that this organism, unique in its temperature range for growth, grew optimally at 15 - 16 C and would not grow at 20 C or higher. Hagen, et al. studied an unclassified red-pigmented marine organism which had similar temperature characteristics.

It has been implied that psychrophiles grow slowly due to the requirement for a low incubation temperature (13). This concept was opened to argument by the growth rate and cell yields obtained with Vibrio marinus as reported by Morita and Haight (24) and Morita and Albright (22). The latter found 24-hour cell yields of 10^9 per ml at 3.0 C and 13×10^{11} per ml at 15.0 C under optimal conditions.

Ingraham (11) has approached psychrophilism from the standpoint of growth at minimum temperatures. These data indicate that for psychrophiles the energy of activation required for growth may be less than for organisms representative of other temperature classes.

Another approach to studies of the psychrophilic phenomenon has

been to elucidate those controls which regulate the maximum temperature of growth. Many investigators have studied this control in various cultures and a number of explanations have been offered. The following are examples.

Rose (31) suggested that accumulation of toxic materials during incubation might be a factor, but found that reduction of the incubation temperature resulted in the full recovery of the culture. It seemed, therefore, unlikely that toxic products were involved in viability loss.

Intra-cellular organization was suggested by Ingraham (11) and Ingraham and Bailey (12) as the probable source of differences in temperature optima and possibly of maximal and minimal growth temperatures.

Nucleic acid lability was shown by Marmur (19) to be physically unrelated to maximal growth temperatures as the melting point (Tm) values of purified DNA for a series of different mesophilic genera were all within the range of 80-95 C.

Hagen and Rose (10) in studies on a psychrophilic Cryptococcus found that at the maximum growth temperature there was an accelerated use of the intra-cellular amino acid pool. This was shown to be related to the loss of ability to synthesize alpha-ketoglutarate at this temperature.

Differences in lipid constitution have been suggested to be involved in the limitation of growth at high temperatures. Kates and Hagen (15)

found, for a species of Serratia, that as the incubation temperature was increased the degree of saturation of fatty acids increased. A similar situation, found to exist in Escherichia coli, was reported by Marr, et al. (20). Kates and Baxter (14) found that the total extractable lipid was approximately equal from mesophilic and psychrophilic strains of Candida. However the psychrophilic strain contained a higher percentage of unsaturated fatty acids.

Growth of a culture is a reflection of the enzymic processes carried on by that culture (31). Consequently, viability loss and failure to grow at high temperatures has been attributed to enzyme denaturation or inactivation. Edwards and Rettger (6) found several enzymes in a species of Bacillus which were inactivated at temperatures just above the maximum for growth. In an investigation by Langridge (16), the enzyme, malic dehydrogenase, which was partially purified from Vibrio marinus, strain MP-1, was shown to be inactivated at temperatures above 20 C. Robison (30) reported that heating Vibrio marinus at inimical temperatures reduced respiration both with and without glucose as substrate. While examining the thermal sensitivity of marine cultures, Zobell and Conn (41) found a definite decrease in oxygen uptake at 30 C compared with the activity at the optimal growth temperatures, and on this basis suggested that the respiratory enzymes were important in the ability to grow at higher temperatures.

Nashif and Nelson (25) showed that although lipase activity in

Pseudomonas fragi was optimal at 40 C, no enzyme was formed above 30 C; this suggested a heat-sensitive enzyme-forming system. Similarly, Upadhyay and Stokes (38), working with both mesophilic and facultatively psychrophilic strains of Escherichia coli, found that formic hydrogenlyase activity was optimal at 30 C and inactive at 45 C in the psychrophilic strain, but active at both temperatures in the mesophile. They concluded that the enzyme-forming system was inactive above 20 C in the psychrophile.

Morita and Burton (23) suggested, for a facultatively psychrophilic strain of Vibrio marinus, that permeability change as well as enzyme lability was a partial reason for the maximum growth temperature of that culture. Hagen, et al. (9) have demonstrated heat-induced lysis in an unclassified psychrophilic marine culture, but stated that lysis occurred two degrees above the temperature at which viability was lost. They also implied that lysis in this culture was enzyme mediated. Robison (30) observed 260 mμ absorbing material in cell supernatants of Vibrio marinus after the cell suspension had been incubated at temperatures above the maximum for growth. A similar observation was made by Hagen, et al. (9).

In full recognition of the manifold temperature effects on varied organisms, further characterization of Vibrio marinus, strain MP-1, was undertaken with a view toward determining the nature of the primary lesions induced by heating at temperatures above 20 C.

MATERIALS AND METHODS

The names and sources are given in the Equipment Appendix for all major pieces of apparatus used. The Chemical Appendix lists chemicals which may not be commonly stocked in the laboratory and their sources. The Reagent Appendix gives the composition and preparation of the reagents used in this research.

The Culture

The marine psychrophile, Vibrio marinus MP-1, isolated by Morita and Haight (24) and taxonomically described by Colwell and Morita (4), was used in these studies. Stock cultures were maintained at 15 C in 2216 E or SDB broth. Hereafter in this thesis, the culture will be referred to as MP-1.

Growth Media

Medium 2216 E was composed of neopeptone (Difco), 5.0 g; yeast extract (Difco), 1.0 g; ferric phosphate, 0.1 g; 750 ml aged sea water, and 250 ml distilled water, pH 7.2-7.4. After boiling, the medium was filtered and the pH readjusted to 7.3. The medium was dispensed into vessels as required and sterilized by autoclaving 15 minutes at 121 C and two atmospheres pressure..

SDB broth was composed of polypeptone (Difco), 5.0 g; yeast

extract (Difco), 3.0 g; Rila salts, 6.0 g; sodium chloride, 21.0 g; glucose, 1.0 g; succinic acid, 0.3 g; and 1,000 ml distilled water, pH 7.3. The medium was dispensed and sterilized as described above. Filtration was not necessary as no precipitate formed on autoclaving.

SDB agar was the same as SDB broth, except the former contained 15 g agar agar (Difco) and 0.5 ml Tween 80 per liter.

RDH broth was composed of polypeptone (Difco), 5.0 g; yeast extract (Difco), 3.0 g; Rila salts, 1.0 g; sodium chloride, 26.0 g; glucose, 1.0 g; and 1,000 ml 0.025 M phosphate buffer at pH 7.3. No filtration was required as precipitation did not occur prior to or after dispensing and autoclaving.

All media and preparative reagents were at all times cooled to refrigeration or incubation temperatures prior to use.

pH Optimum for Growth

RDH broth was prepared at pH 6.2, 6.7, 7.0, 7.2, 7.4, 7.6, 7.9, and 8.5. The broth was dispensed in 10.0 ml aliquots into 18 x 150 mm test tubes and sterilized by autoclaving. The tubes were inoculated with 0.1 ml of a 12-hour culture grown in RDH broth at 15 C. Growth was determined by measuring optical density (OD) at 625 mμ in the spectrophotometer at inoculation time and after 14 hours of incubation at 15 C.

Growth Rate

The rate of growth of MP-1 in medium 2216 E was determined by following the OD increase with time of incubation. One hundred ml portions of medium were dispensed into 500-ml Erlenmeyer flasks, each fitted with a sidearm nephelometer tube. These were inoculated from a 12-hour 15 C culture (one percent by volume), and incubated at 15 C with reciprocal shaking (200 strokes per minute with a 2.54 cm stroke) in the PsychroTherm. OD readings were made at 30 minute intervals at 525 mμ by inserting the nephelometer sidearm into the spectrophotometer.

Halotolerance

Medium 2216 E was prepared at salinities ranging from zero ‰ to 70 ‰ using Rila salts instead of aged sea water. The broth was dispensed (10.0 ml into 15 x 100 mm screw capped test tubes) and autoclaved. Each tube was inoculated with one drop of a 12-hour culture grown in medium 2216 E at 27 ‰. Incubation was carried out at 15 C for 144 hours during which visible turbidity was checked every 24 hours. Growth was recorded as positive or negative.

Nutrient and Thermal Effects on Viability

A one percent inoculum by volume of a 12-hour culture grown at

15 C was introduced into tubed 2216 E medium. These tubes were placed into the equilibrated polythermostat and one tube at each temperature was removed after 1.25, 3.0, 6.25, and 9.0 hours of heating. After heating, the tubes were immediately incubated at 15 C in the PsychroTherm. Growth was estimated visually at 24, 48, 72, 96, and 168 hours.

Quantitative studies on thermally-induced death were carried out on cells grown in SDB broth from a one percent by volume inoculum. Incubation conditions were 15 C for 24 hours with shaking in the PsychroTherm. After growth, the culture was aseptically divided into two equal portions. Both were aseptically centrifuged in the RC-2 at 0 C at 10,400 x g for 15 minutes. The cell buttons were aseptically suspended, each to its original volume; one (without nutrients) in sterile 27‰ Rila sea water (RSW), the other (with nutrients) in sterile SDB broth. Each of these preparations was aseptically divided into two equal portions. One set (with and without nutrients) was incubated at 31 C; the other set was incubated at 25 C. At timed intervals one-ml aliquots were removed from each preparation and immediately carried through a series of ten-fold dilutions. The dilution broth was composed of proteose peptone (Difco), 5.0 g; Rila salts, 5.0 g; sodium chloride, 22.0 g; yeast extract (Difco), 3.0 g; distilled water, 1,000 ml, at pH 7.2. Triplicate 0.1-ml aliquots were immediately removed from each dilution and spread plates made on SDB agar. The entire

dilution and plate-spreading procedures required less than 30 minutes to complete. The plates having between 30 and 300 colonies were counted. The percent viable cells remaining was calculated.

Preparation of MP-1 DNA

A carboy containing 12 liters of 2216 E plus 0.1 percent glucose was inoculated (ten percent by volume) from a culture grown in the same medium at 15 C for 12 hours. It was incubated at 15 C for 24 hours with continuous air-sparging during which foaming was controlled by occasional treatment with anti-foam spray. Harvest was carried out at 0 C by continuous flow centrifugation at 27,000 x g. The collected cells were immediately frozen and kept until needed. All further procedures were carried out at 15 C or lower. The following method was essentially that of Marmur (19).

Frozen cells (3.7 g) were thawed and mixed thoroughly with 25.0 ml saline-EDTA. After the addition of 5.0 ml sodium lauryl sulfate, the mixture was actively stirred until the viscosity had noticeably increased, which was usually instantaneous. Five M sodium perchlorate (7.5 ml) was then added to bring the perchlorate concentration to 1.0 M. After thorough mixing an equal total volume (37.5 ml) of chloroform-isoamyl alcohol reagent (CI) was added and the mixture was rapidly shaken for 15 minutes. Centrifugation at 0 C for ten minutes at 1,085 x g was used to break the emulsion and to allow the

phases to separate. The clear supernatant was removed by pipette and cooled in an ice bath. Then two volumes of cold 95 percent ethanol were carefully layered over it. The fine white threads of nucleic acid formed at the interface were wound onto a small glass rod. The collected windings were pressed dry on the side of the beaker and were dissolved in 15 ml dilute saline-citrate (DSC). After solution had been accomplished, 1.5 ml concentrated saline-citrate (CSC) was added to increase the concentration of saline-citrate to normal, i.e., to that of normal saline-citrate (NSC). Three successive deproteinizations were carried out with CI after which the nucleic acids were collected and treated as described above. After the last deproteinization, the nucleic acids were dissolved in 0.5 supernatant volumes of NSC. Then 0.3 ml 0.2 percent ribonuclease (RNase) solution was added. The resultant mixture was incubated at 37 C for 30 minutes to digest the contaminating ribonucleic acid (RNA). Deproteinization was again performed and the desoxyribonucleic acid (DNA) was collected by winding. After dissolving the DNA in 9.0 ml DSC, 1.0 ml acetate-EDTA was added, and while stirring rapidly, 0.60 volumes of cold isopropanol were added dropwise to the vortex. A gel phase was noted when 0.50 volumes of isopropanol had been added. Thread formation was complete at 0.60 volumes. The DNA was collected by centrifugation for 15 minutes at 0 C and 27,000 x g. After decantation, the precipitate was dissolved in 10.0 ml NSC which yielded a

sparkling clear DNA solution. This preparation was used for all melting point determinations and was stored in the refrigerator.

Determination of DNA Melting Temperature

Both salmon sperm and MP-1 DNA dissolved in NSC were diluted with NSC to an OD of 0.2 - 0.4 at 260 mμ as determined with the DU. These preparations were used for melting point (Tm) studies.

The kinetics of melting were determined by reading the OD of quick-cooled aliquots of DNA heated at 95 C for various lengths of time. Quick cooling was accomplished by placing the samples in ice for two minutes.

The Tm values for MP-1 and salmon sperm DNA were obtained by reading the OD of samples immediately after heating ten minutes at the desired temperature. The OD values were corrected for expansion. The relative absorbance and Tm values were calculated according to the method of Marmur (19).

The percent guanine-cytosine (G-C) and adenine-thymine (A-T) in DNA was calculated by the equation of McDonald et al., (21) given as follows:

$$\text{Percent (G-C)} = (\text{Tm}-69)\ 2.439$$

Percent Lipid Content of MP-1

Two Fernbach flasks containing 300 ml SDB broth were inoculated

(one percent by volume) from a 12-hour culture grown at 15 C. Incubations were at 4 C and 15 C for 36 and 14 hours, respectively. The shaking rates were equal. In the late log phase of growth, cells were harvested by centrifugation at 0 C for 15 minutes at 10,400 x g, washed once with 27 ‰ RSW, and suspended in 27 ‰ RSW. These suspensions were then lyophilized.

Known weights of lyophilized cells were extracted by batch-wise shaking with three 10.0 ml volumes of chloroform-methanol solvent (CM), and the extracts were combined. Washing of the extracts was accomplished by shaking them with 0.2 volumes of 0.73 percent sodium chloride in separatory funnels. After phase separation the upper phases were removed by siphon and the surface of the lower phases were rinsed twice with pure-solvent-upper-phase (PSUP) to remove salts and contaminants. The washed preparations were then evaporated to dryness in tared flasks under a stream of nitrogen gas. The weights of the residues were determined and the percent extractable lipid was calculated for both 4 C and 15 C grown cells. The above methodology was similar to that of Folch, et al. (7).

Method for Heat Shocking

Mass culture in SDB broth and harvesting were accomplished as described in the preparation of DNA. After the cells had been washed twice with 27 ‰ RSW, they were suspended in 27 ‰ RSW to a

volume such that a one to 100 dilution gave an OD at 600 mμ of 0.40 ± 0.01 as determined in the spectrophotometer. The undiluted cell suspension was then dispensed (ten ml per tube), placed in the equilibrated polythermostat and incubated as desired. After incubation the cells were removed by centrifugation at 0 C and 27,000 x g for ten minutes. The supernatants were decanted carefully and stored in the refrigerator until analyzed. The protein concentration was determined immediately.

Protein Estimation

One-ml aliquots of the various supernatants were mixed with four ml aliquots of Biuret reagent and allowed to stand at room temperature for 30 minutes. The OD was determined at 560 mμ with the spectrophotometer. The concentration of protein was calculated from a standard curve prepared simultaneously with bovine serum albumin. This method was essentially that given by Layne (17).

RNA Estimation

The method used was a modification of Schneider's (33). The sample was deproteinized by adding an equal volume of ten percent trichloroacetic acid (TCA) and heating for ten minutes in the steamer. Clear supernatants, protein free, were obtained by filtration through TCA-washed Whatman Number One filter papers.

Four ml of fresh orcinol reagent were added to one ml of appropriately diluted sample in screw capped tubes. The tubes were steamed 15 minutes to develop the color. After cooling to room temperature the OD values were obtained at 660 mμ on the spectrophotometer. RNA concentrations were calculated from a standard curve prepared simultaneously with yeast RNA.

DNA Estimation

The method of Burton (2) was used as the basis for this procedure. Deproteinization of the sample was accomplished as described under RNA estimation.

One ml of sample was mixed with 0.5 ml 0.5 N perchloric acid in screw capped tubes. If cloudiness appeared the tubes were steamed for 15 minutes or until no turbidity was apparent. After cooling, three ml of fresh diphenylamine reagent were added, and the tubes were incubated for 18 hours at 30 C. OD values were obtained at 600 mμ in the spectrophotometer. DNA concentrations were calculated from a standard curve prepared simultaneously with salmon sperm type III DNA.

Amino Acid Estimation and Analysis

This method is similar to that of Spies (36). Deproteinization of the sample material was accomplished as described above.

Two ml of ninhydrin reagent were added to one ml of appropriately diluted sample and, after a 20 minute steaming period, the tubes were cooled to room temperature. Five ml of diluent solution were added to each tube. OD values were determined at 575 mμ on the spectrophotometer. The amino acid concentrations were calculated from a standard curve prepared simultaneously with L-alanine.

The individual amino acids present in the deproteinized supernatants were analyzed on an automatic amino acid analyzer by Dr. R. R. Becker.

The percent increase of the various constituents in the heat-shock supernatants was calculated as follows:

$$\frac{C_x - C_o}{C_o} \times 100 = \text{percent increase}$$

where: C_x = amount per ml at time t

C_o = amount per ml at time 0

Characterization of RNA in Heat-Shock Supernatant

The cells were grown in RDH broth for 12 hours at 15 C with shaking, and were harvested, washed, and suspended in 27 ‰ RSW as described under Method for Heat Shocking. For this study, the conditions for heat shocking (25 C for 60 minutes) were selected since RNA appeared in the supernatant without very much interference by protein or DNA.

The preparation to be heat shocked was placed in a 250-ml Erlenmeyer flask which was suspended in a 25 C water bath. Samples were removed before heating (time 0) and after heating 60 minutes, after which centrifugation at 0 C for 15 minutes at 27,000 x g was carried out. Both supernatants were stored in the refrigerator.

A third sample was prepared by subjecting a five-ml portion of suspended cells (27 ‰ RSW) to high frequency sound waves for four 30-second pulses in the Sonic Oscillator. After centrifugation for 15 minutes at 27,000 x g at 0 C, the supernatant (cell lysate) was stored in the refrigerator.

Salmon sperm type III DNA and yeast RNA dissolved in distilled water served as controls for Sephadex G-25 column chromatography.

Sephadex column chromatography was carried out with a Pharmacia column gravity-packed with Sephadex G-25 find bead gel to bed dimensions of 2.54 x 32.7 cm, using distilled deionized water as the solvent and eluent. The elution peaks were monitored continuously by an ISCO monitor. Appropriate sample volumes were washed into the column surface and a hydrostatic head of one inch above the gel surface was applied. The flow rate was 100 ml per hour. Dextran 2000 was used to determine the void volume of the column.

RNA Analyses of Heat-Shock Supernatant and Cell Lysate

Five-ml aliquots of the 25 C-60 minute heat-shock supernatant and

of the cell lysate were mixed with an equal volume of cold ten percent TCA. The precipitates were removed by centrifugation at 0 C for 15 minutes at 27,000 x g. The supernatants were decanted, increased to 10.0 ml with five percent TCA, and saved (cold supernatants). After washing the precipitates once with cold five percent TCA, they were suspended in 10.0 ml of five percent TCA and heated in the steamer for 30 minutes to solubilize polymeric RNA. The residues were discarded after centrifugation and the supernatants were saved (hot supernatants). This hot and cold TCA treatment was essentially that used by Duncan (5). A similar method was given by Schneider (32). Orcinol determinations were carried out on these supernatants to determine the distribution and levels of orcinol-reacting materials in these extracts. The ratios of RNA found, based on yeast RNA as the standard, were calculated.

Analysis of Supernatants for Enzyme Activity

The preparation of the cells and the heat shocking procedure were as described in Method for Heat Shocking except that the suspending menstra were 75 ‰ RSW and 75‰ ammonium sulfate Rila salts (ARS). It was hoped that the use of the higher salinity values would stabilize enzymic activity in the supernatants. The time of heat shocking was 30 minutes.

The determination of malic dehydrogenase activity and the

calculation of units were according to the method of Ochoa (26). Activity was assayed at 20 C by following the decrease in OD at 340 mμ when reduced nicotinamide adenine dinucleotide (NADH) was oxidized using oxalacetic acid (OAA) as substrate. The reaction mixtures contained in 3.0 ml: 1.6 μmoles OAA, 0.2 μmoles NADH, 520 μmoles tris (hydroxymethyl) aminomethane-sulfate buffer at pH 7.4, and 0.1 ml supernatant of varying protein concentration. All assays were performed on a DU equipped with thermospacers.

RESULTS

Before studies on the biochemical basis of obligate psychrophilism were initiated, it was necessary to characterize this culture microbiologically. The optimum and maximum temperatures for growth had been shown earlier by Morita and Haight (24) to be 15-16 C and slightly less than 20 C respectively.

The optimum pH for growth under laboratory conditions is shown in Figure 1. Good growth occurred throughout the range of 6.7-8.5, with the maximum at pH 7.3-7.4.

Figure 2 is a plot of growth (measured by OD) versus time of incubation at 15 C. A lag of four hours was followed by the log phase of growth which continued for about six hours. After ten hours of incubation, the culture appeared to enter the stationary phase of growth. Note that rapid or log phase of growth was relatively short, and was completed within 12 hours after inoculation.

The range of salinity tolerated by this organism is shown in Table I. Good growth was apparent within 24 hours incubation at salinities 15 ‰ through 50 ‰ and only after extended incubation did growth appear at salinities 10 ‰ and 55 ‰. The growth at the extreme salinities appeared to be granular and clumped rather than smooth and dispersed throughout the tube as with less inimical salinities. This organism appeared to have a rather wide salinity

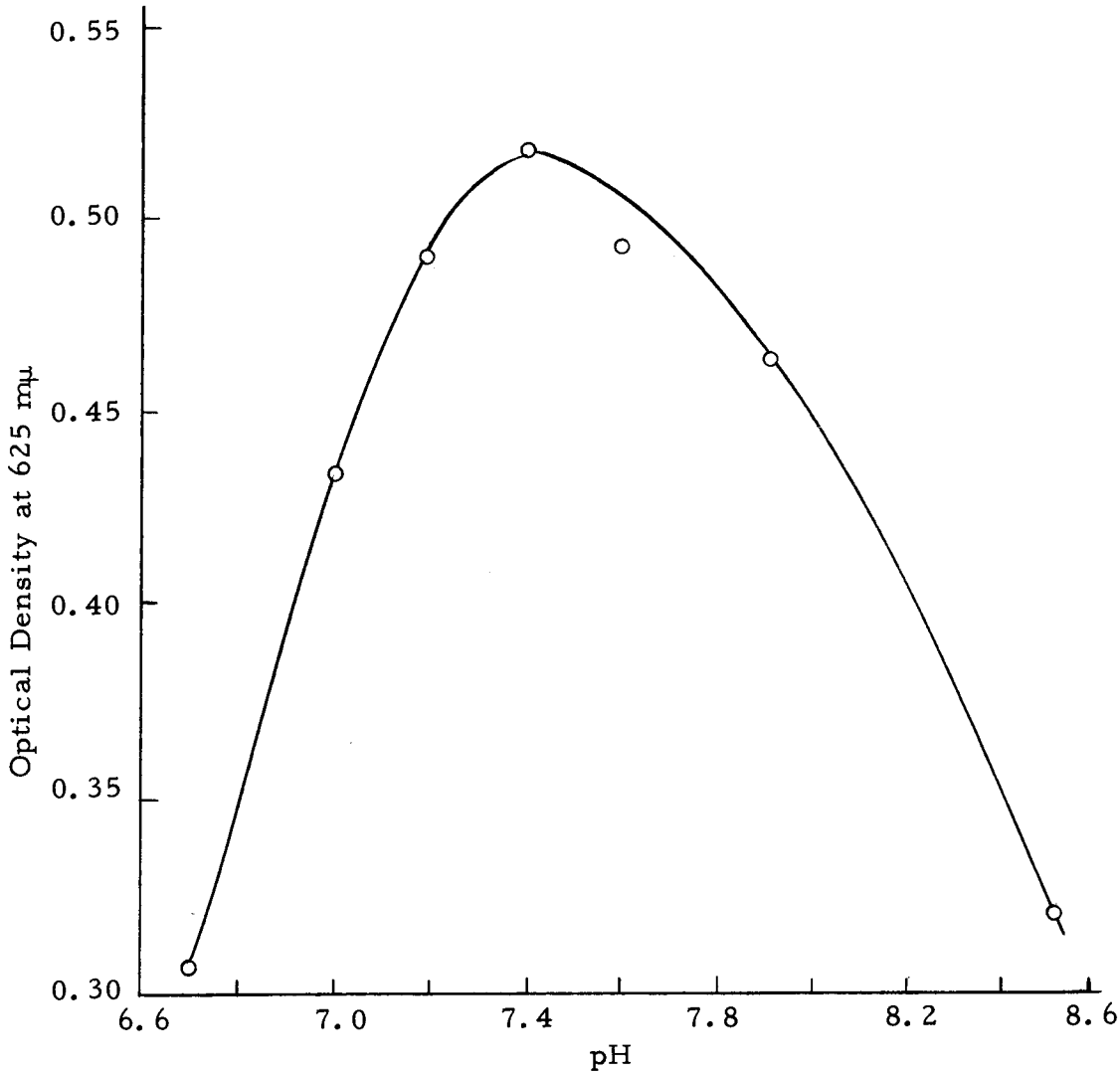

Figure 1. The effect of pH on growth of Vibrio marinus MP-1. RDH broth dispensed in 10-ml aliquots into 18 x 150 mm test tubes was cooled and inoculated with 0.1 ml of a 12-hour culture of MP-1. Optical density at 625 mμ was measured immediately after inoculation and after 14 hours incubation at 15 C. OD values were corrected for the initial reading.

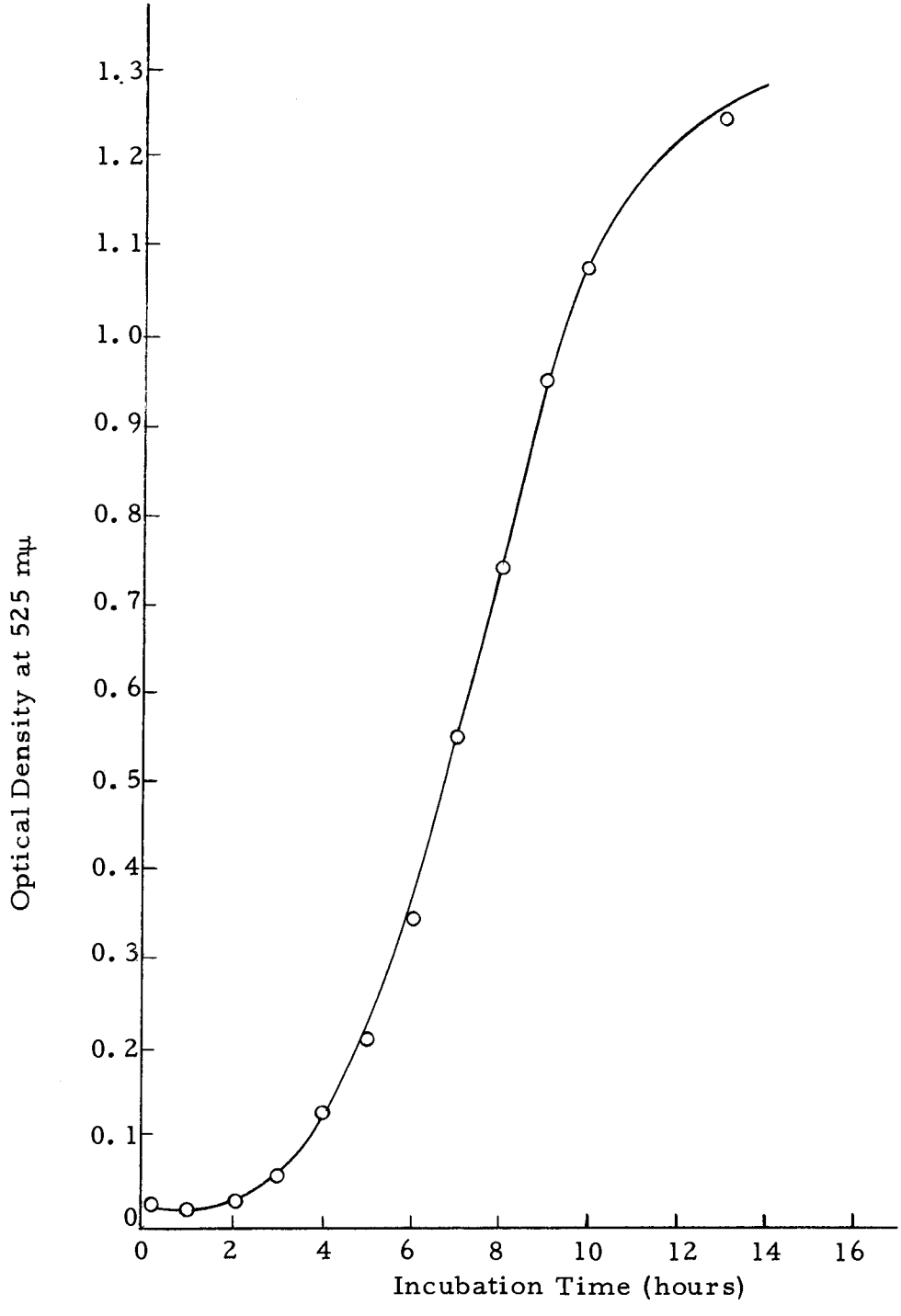

Figure 2. The growth rate of Vibrio marinus MP-1 with aeration. Erlenmeyer flasks (500-ml) fitted with side-arm nephlometer tubes containing 100 ml of medium 2216 E, pH 7.2, were cooled and inoculated with 1.0 ml from a 12-hour culture of MP-1. Optical density readings at 525 mμ were taken initially and at 30 minute intervals. Aeration was accomplished by shaking at 200 strokes per minute with a 2.54 cm stroke. Incubation was at 15 C.

TABLE I. HALOTOLERANCE FOR GROWTH OF VIBRIO MARINUS MP-1.

Salinity (‰)	Visible Turbidity			
	0 hours	24 hours	48 hours	72 hours*
0	-	-	-	-
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
10	-	-	+	+
15	-	+	+	+
20	-	+	+	+
30	-	+	+	+
35	-	+	+	+
40	-	+	+	+
45	-	+	+	+
50	-	+	+	+
55	-	-	+	+
60	-	-	-	-
65	-	-	-	-
70	-	-	-	-

*A series of 2216 E media of varying salinities was inoculated from a 12-hour culture grown at salinity 27 ‰. Incubation was carried out for the stated times at 15 C. Visible turbidity was recorded at 24-hour intervals.

tolerance for growth.

Table II shows that incubation for various periods of time at temperatures at which the organism will not grow may or may not render

TABLE II. INFLUENCE OF TIME AND TEMPERATURE ON THE SURVIVAL OF VIBRIO MARINUS MP-1.

Temperature (C)	Heating Time (hours)			
	1.25	3.0	6.25	9.0
19.0	+	+	+	+
21.0	+	+	+	+
23.0	+	+	+	+
25.0	+	+	+*	+*
27.0	+	+	+**	+**
28.8	+*	+*	-	-
30.8	+*	+**	-	-
32.7	-	-	-	-
34.8	-	-	-	-
36.9	-	-	-	-

*48-hour observation of visible turbidity.

**96-hour observation of visible turbidity. Further observations beyond 96 hours did not change the above results.

After exposure to the various temperatures and times the tubes of 2216 E broth were incubated at 15 C up to 168 hours. Inoculation was at one percent by volume from a 12-hour culture.

the culture non-viable. The results show that the inoculum was not totally destroyed when heated for 9.0 hours at 27.0 C but was rendered nonviable within 1.25 hours at 32.7 C. This suggested that the culture had a rather low tolerance to heat, even when adequately supplied with nutrients.

Robison (30) and Haight (8) have shown this bacterium to have a high level of endogenous reserve. In view of this, the effect of

nutrients on viability retention was investigated. Figure 3 shows the results. Viability was rapidly lost at 31.0 C both with and without nutrients, whereas nutrient presence appeared to allow the cells to remain viable at 25.0 C.

Figure 4 shows that purified salmon sperm DNA completely melted at 95 C within ten minutes heating time, as determined after the samples had been quickly cooled. It was tacitly assumed, in the case of the MP-1 DNA curve shown in Figure 5, that these same melting kinetics would apply also when the MP-1 samples were read hot.

Salmon sperm DNA melted at 82 - 92 C whereas MP-1 DNA melted from 86 - 92 C. The midpoints of these curves, the Tm values, were 87.0 C and 89.0 C, respectively. The percents guanine-cytosine and adenine-thymine for MP-1 were 48.8 and 51.2, respectively.

Studies of the lipids of MP-1 indicated that the incubation temperature did not influence the extractable lipid content; it remained at 4.6 percent by weight for both 4 C and 15 C incubation temperatures. Attempts to separate the lipid components by thin layer chromatography met with failure due to lack of an adequate and consistent solvent system.

Robison (30) found that cells of MP-1, when heated at temperatures above the maximum for growth, appeared to shrink and release 260 mμ absorbing material into the surrounding menstrum. This phenomenon, closely related to thermally-induced viability loss, was

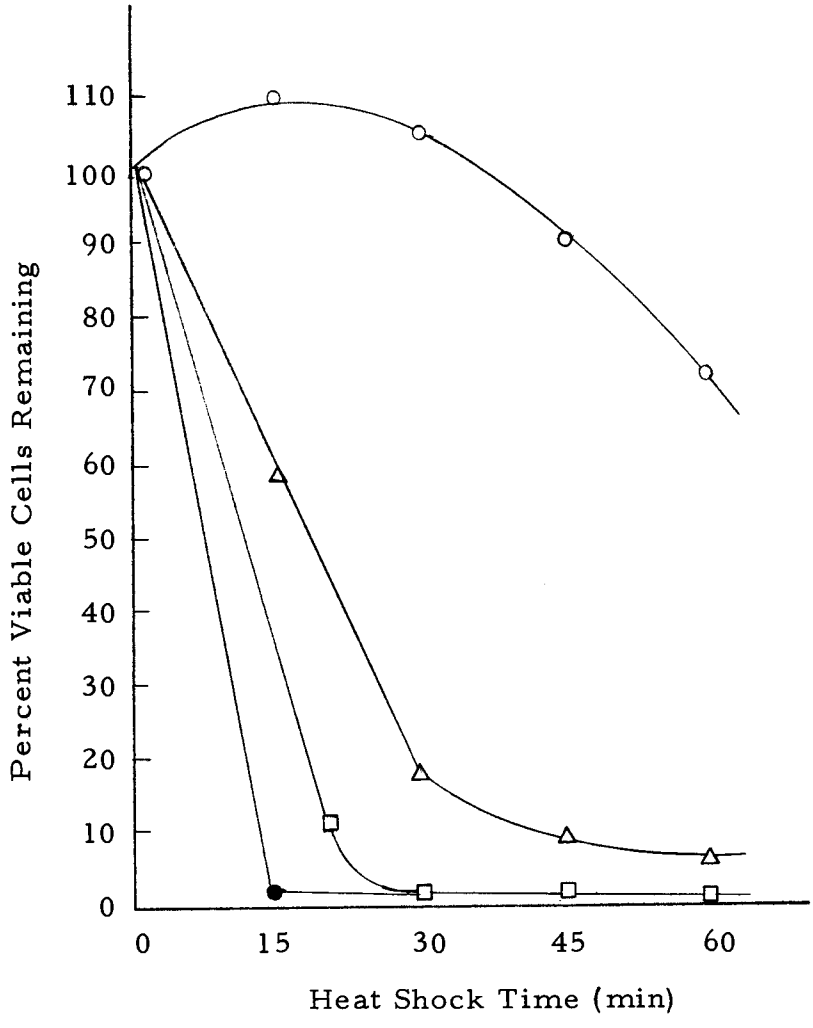

Figure 3. The effect of nutrients on viability retention of Vibrio marinus MP-1. Log phase cells were suspended in cold Rila sea water, salinity 27 ‰, and in 2216 E broth. Aliquots of each were removed during heat shocking, diluted, and plated on SDB agar. The percent viable cells remaining were calculated.

- o indicates 25 C with nutrients
- Δ indicates 25 C without nutrients
- □ indicates 31.0 C with nutrients
- ● indicates 31.0 C without nutrients.

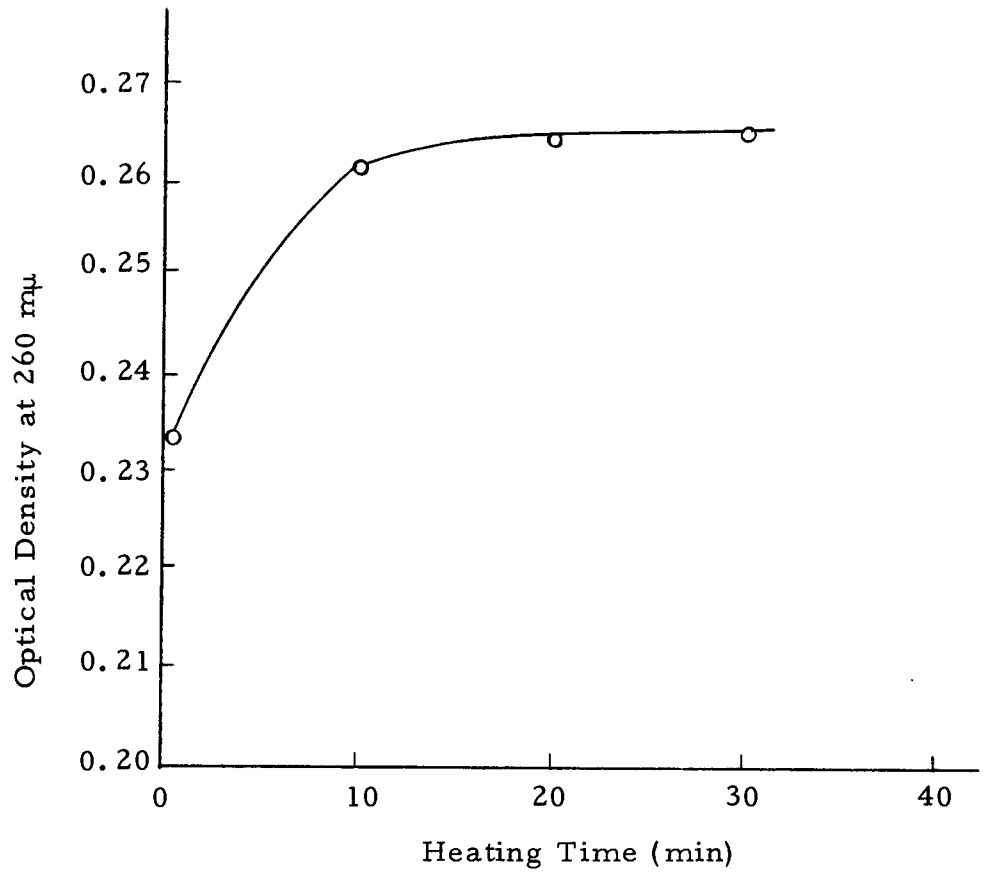

Figure 4. The melting kinetics of salmon sperm type III DNA. Aliquots of a salmon sperm type III DNA preparation were heated for different time periods at 95 C followed by quick cooling. The OD was read at 260 mμ.

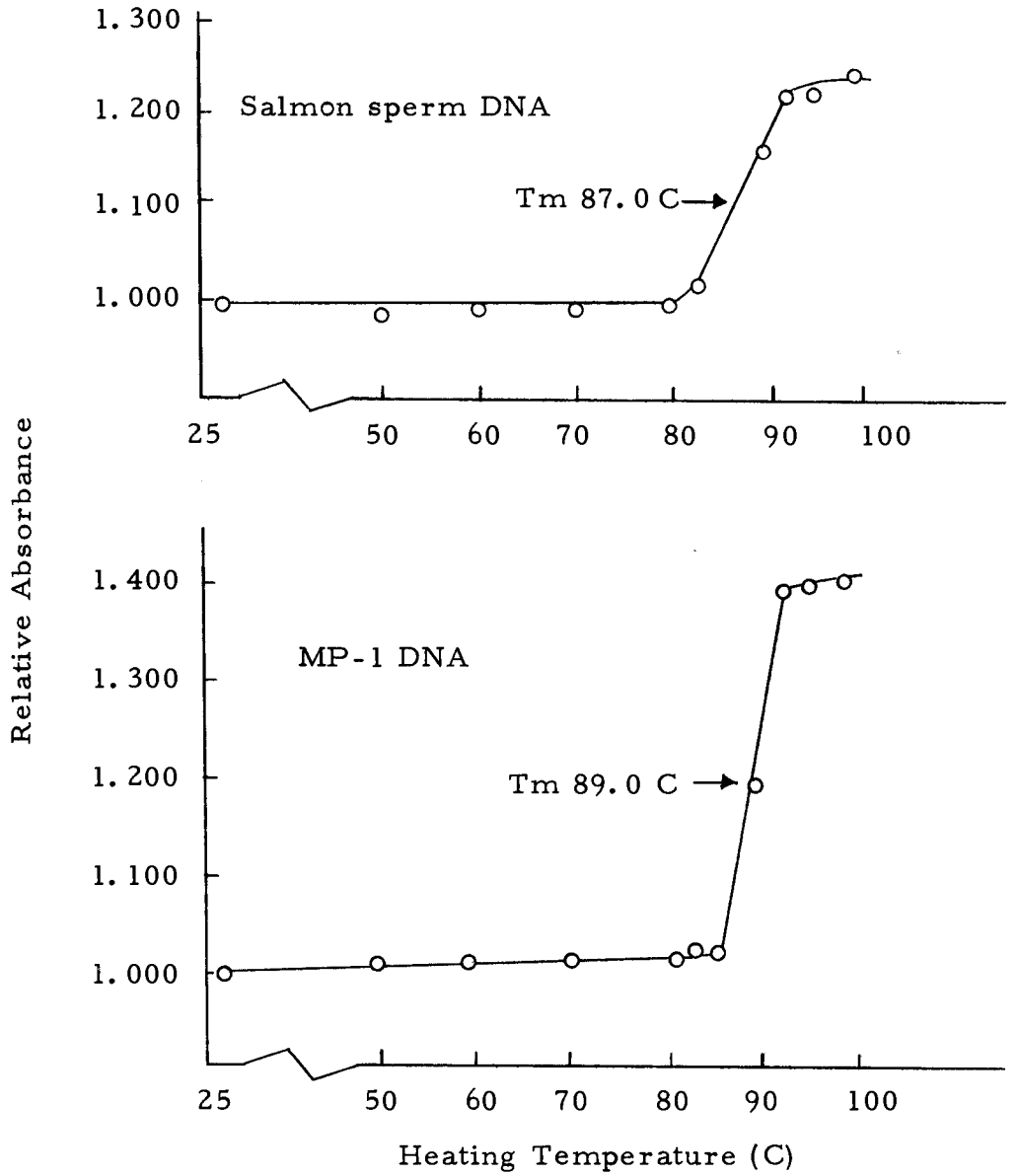

Figure 5. The melting curves (Tm) of salmon sperm type III DNA and Vibrio marinus MP-1 purified DNA. Aliquots of DNA were heated for ten minutes at the stated temperature and the OD at 260 mμ was read immediately. Relative absorbance and Tm values were calculated by the method of Marmur (19).

investigated further. Figures 6 through 8 show the kinetics of release of protein, DNA, RNA, and amino acids at 15.0 C, 22.3 C, and 29.7 C, respectively. Figure 6 illustrates the fact that very little, if any, of these materials leak out of the cells within 60 minutes at 15.0 C. At 22.3 C (Figure 7) these substances do not appear in the supernatant until the cells have been heated at least 45 minutes. The rates of leakage were different; i.e., protein leaked out at a rate greater than RNA, DNA, or amino acids. In fact, protein > RNA > DNA > amino acids. This same sequence was noted at 29.7 C (Figure 8). In this case, leakage appeared after only 15 minutes heating.

As the mechanism of constituent release was unknown, the chemical nature of the supernatant materials was of interest. Consequently the individual amino acids released were determined, as were the kinetics of amino-acid release (Figure 9). At 15.0 C the amino acids appeared to be slowly released, whereas at 24.1 C rapid leakage started at 15 minutes. Heating at 31.6 C caused the immediate leakage of amino acids, but the leakage rate was drastically decreased after 30 minutes.

The individual amino acid make-up of three samples, denoted by the arrows on Figure 9, was determined on an automatic amino acid analyzer. The results are shown in Table III. Note that the basic, acidic, and polar amino acids all tended to increase in level as the time and temperature of heat shock increased, whereas the levels of

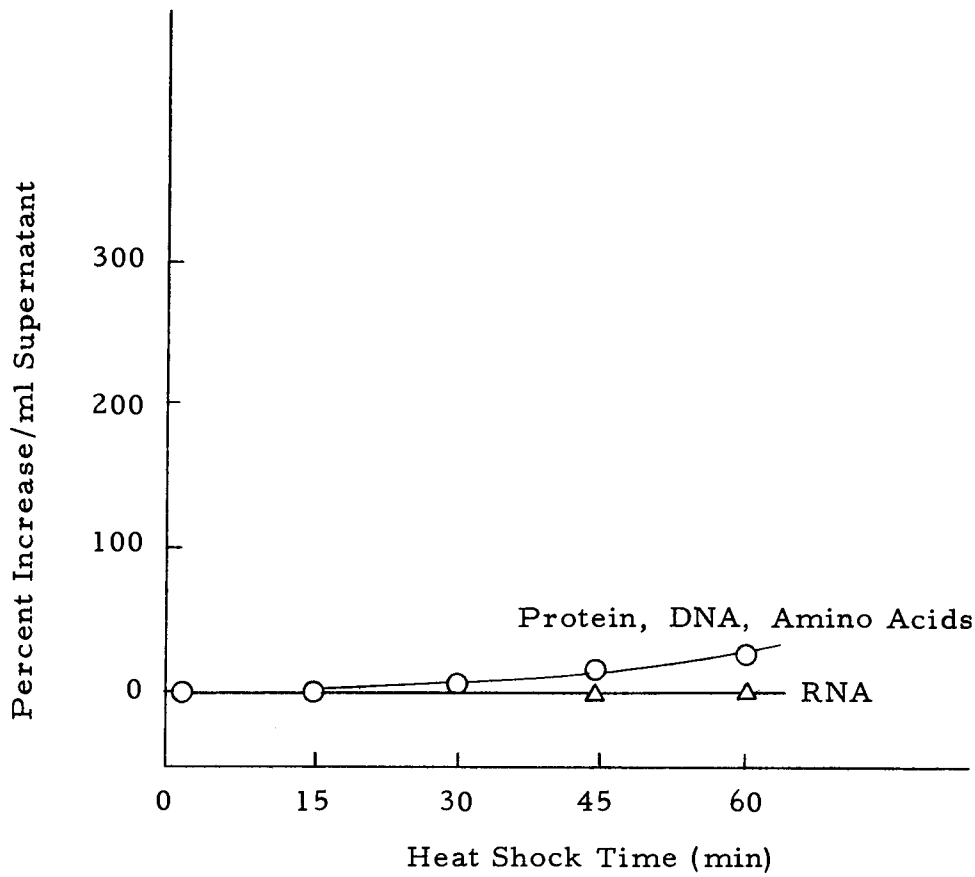

Figure 6. Heat-induced leakage from Vibrio marinus MP-1 at 15 C. Log phase cells were washed and suspended in cold Rila sea water, salinity 27 ‰. Heating was carried out for the stated time intervals in the polythermostat after which the cell debris was removed by centrifugation at 0 C. The supernatants were decanted and stored in the refrigerator until analyzed. The methods of analysis are given in the Materials and Methods section.

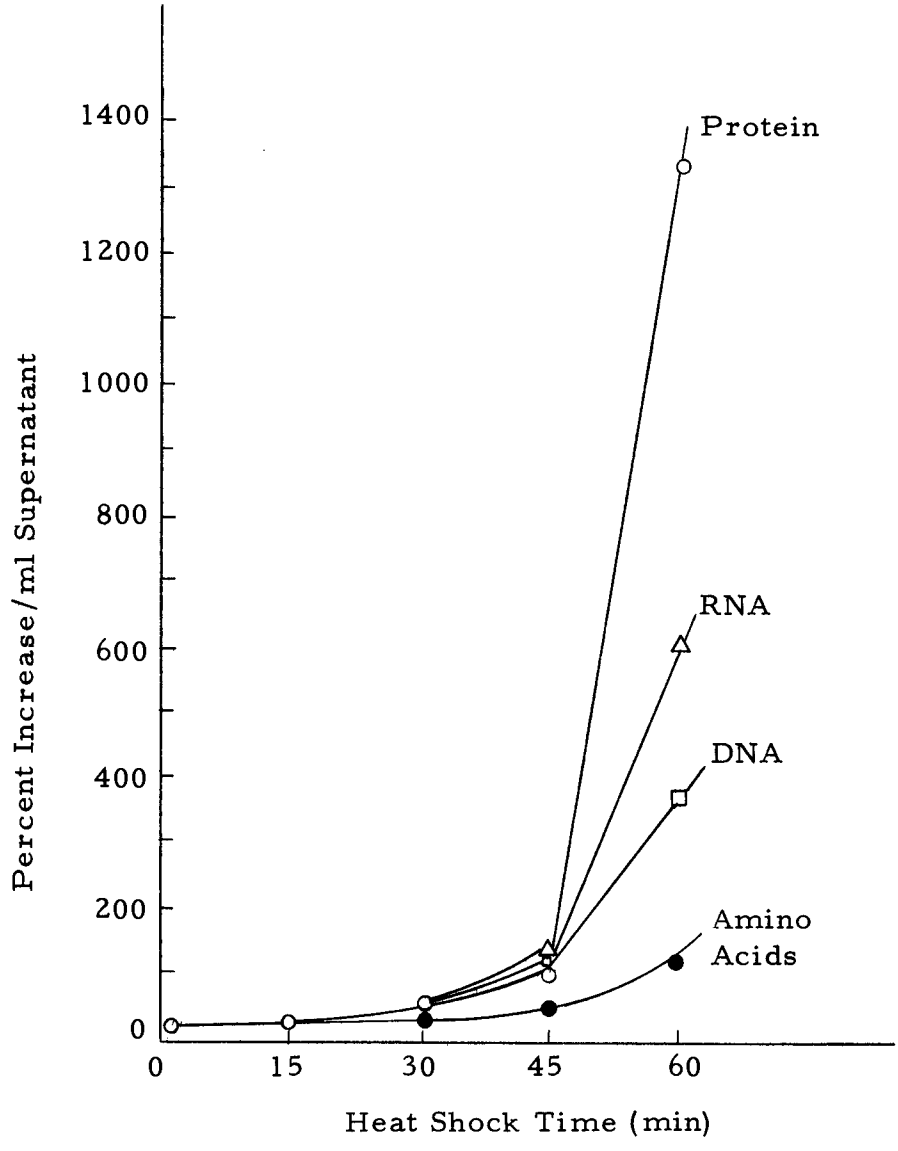

Figure 7. Heat-induced leakage from Vibrio marinus MP-1 at 22.3 C. Log phase cells were washed and suspended in cold Rila sea water, salinity 27 ‰. Heating was carried out for the stated time intervals in the polythermostat after which the cell debris was removed by centrifugation at 0 C. The supernatants were decanted and stored in the refrigerator until analyzed. The methods of analysis are given in the Materials and Methods section.

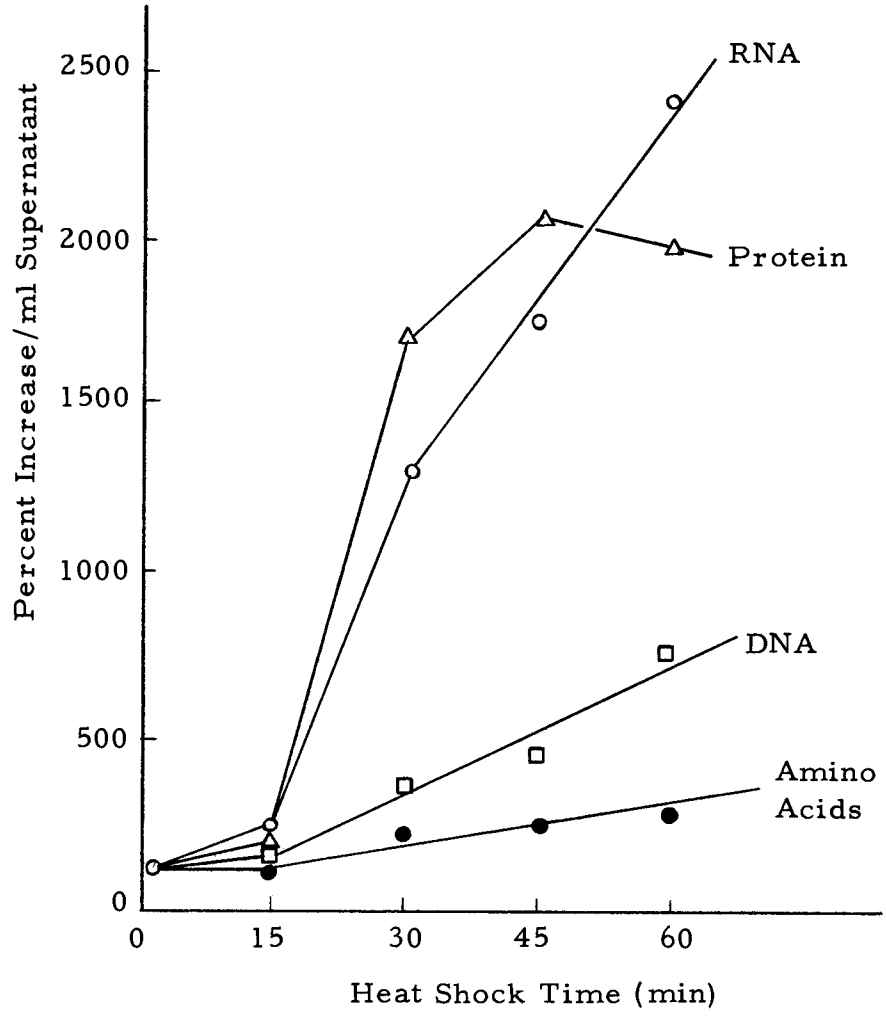

Figure 8. Heat-induced leakage from Vibrio marinus MP-1 at 29.7 C. Log phase cells were washed and suspended in cold Rila sea water, salinity 27 ‰. Heating was carried out for the stated time intervals in the polythermostat after which the cell debris was removed by centrifugation at 0 C. The supernatants were decanted and stored in the refrigerator until analyzed. The methods of analysis are given in the Materials and Methods section.

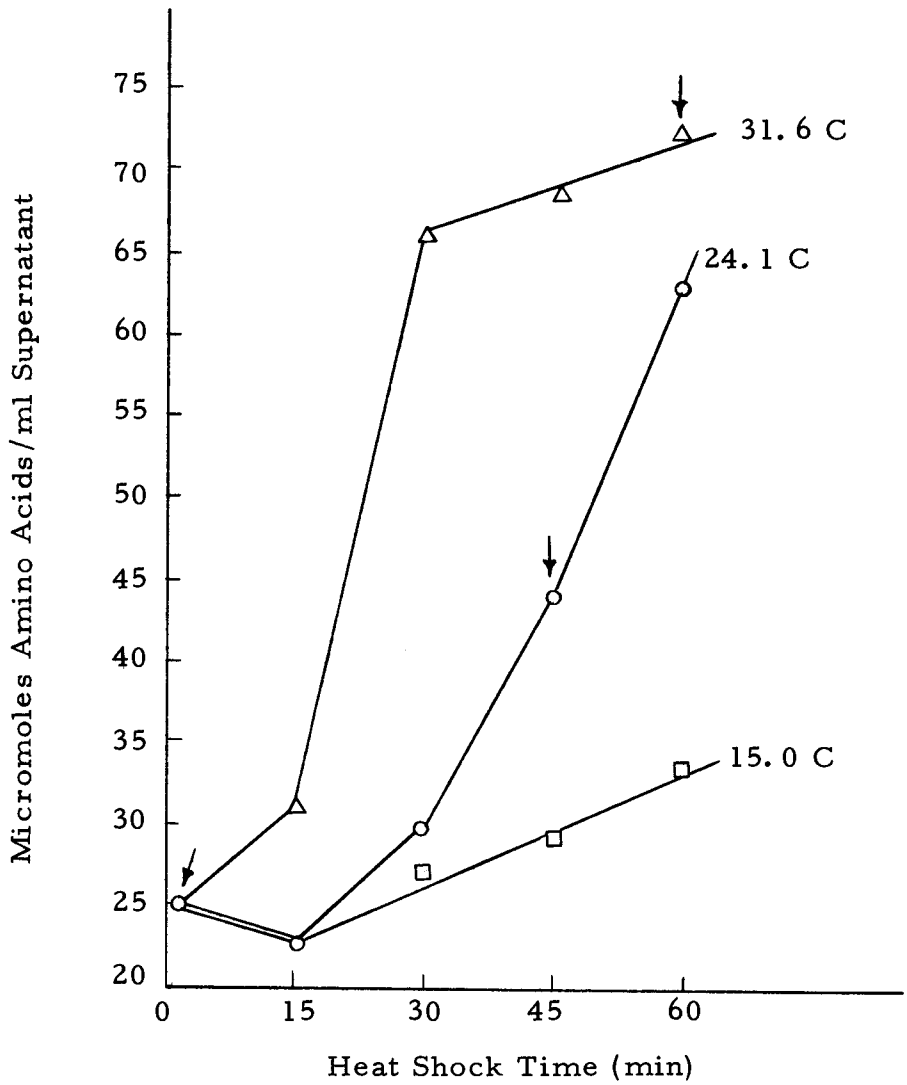

Figure 9. Heat-induced leakage of amino acids from Vibrio marinus MP-1. Log phase cells were washed and suspended in cold Rila sea water, salinity 27 ‰. Heating was carried out for the stated time intervals and temperatures in the polythermostat after which cell debris was removed by centrifugation at 0 C. The supernatants were decanted and deproteinized with ten percent TCA. The amino acid levels were determined by the method given in the Materials and Methods section.

TABLE III. AMINO ACID ANALYSIS OF DEPROTEINIZED SUPERNATANTS FROM HEAT-SHOCKED VIBRIO MARINUS MP-1.

Amino Acid	0 min.	45 min @ 24.1 C	60 min @ 31.6 C
Lysine	.106	.281	.250
Histidine	Trace	Trace	.018
Arginine	0	0	.021
Aspartic	.144	.410	.470
Glutamic	3.270	3.390	4.320
Threonine	.042	.039	.064
Serine	.051	.063	.098
Glycine	.026	.066	.385
Cystine/2	Trace	Trace	.111
Proline	*	*	*
Alanine	1.490	1.240	1.060
Valine	.520	.345	.318
Methionine	.157	.108	.090
Isoleucine	.313	.215	.200
Leucine	.200	.147	.155
Tyrosine	.127	.082	.087
Phenylalanine	.274	.172	.164

*Proline peak obscured by glutamic acid peak.

The deproteinized supernatants were prepared as for Figure 9. Amino acid analysis was accomplished on a Beckman automatic amino acid analyzer.

non-polar amino acids decreased with increased temperature and time of heat shock. Also, in this method of analysis the proline peak was obscured by the very high level of glutamic acid.

The chemical nature of the RNA released by heat shocking for 60 minutes at 25 C was determined by two methods, Sephadex

chromatography and TCA fractionation. Figure 10 shows the elution patterns obtained when elution was monitored continuously by a 254 mμ monitoring cell. Dextran 2000, a commercially available dextran of 2,000,000 molecular weight, was used to determine the location of a totally excluded peak, as well as the void volume of the column. Salmon sperm DNA was totally excluded as expected, whereas yeast RNA appeared to have a small fraction which was not excluded. The cell lysate also contained both excludable and non-excludable materials as did the 25 C - 60 minute heat-shock supernatant. The supernatant from unheated cells contained very little 254 mμ absorbing material, none of which was excludable.

Table IV shows the distribution of orcinol-reacting material in the 25 C - 60 minute supernatant and in the cell lysate. Cold TCA precipitated the polymeric materials without degradation, whereas hot TCA degraded polymeric nucleic acids sufficiently to render them soluble. The data, using this technique, indicated that in the cell lysate the majority of the orcinol-reacting material was found in the cold TCA precipitatable material (therefore polymeric), whereas in the 25 C-60 minute supernatant the reverse was true. No analysis of DNA was attempted.

Since protein leakage preceded all other known constituents, the kinetics of this process were investigated (Figure 11). At both 15.0 C and 22.3 C, very little protein was released during heat shocking,

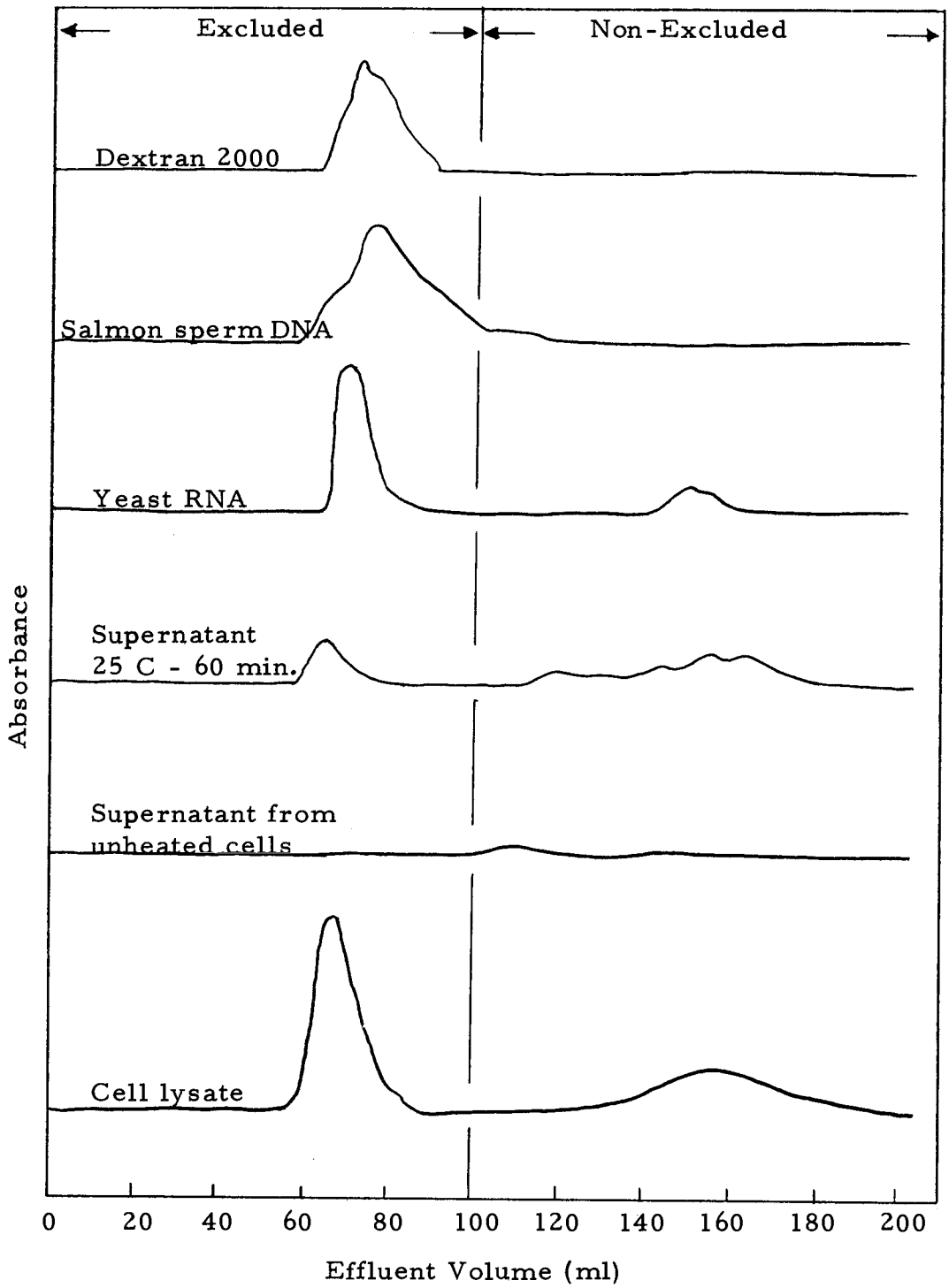

Figure 10. Sephadex G-25 elution patterns of cell lysate and heat-shock supernatant from Vibrio marinus MP-1. The various cell extracts and control materials were prepared according to the Materials and Methods section. Distilled deionized water was used as the eluent and the effluent was continuously and automatically monitored at 254 mμ.

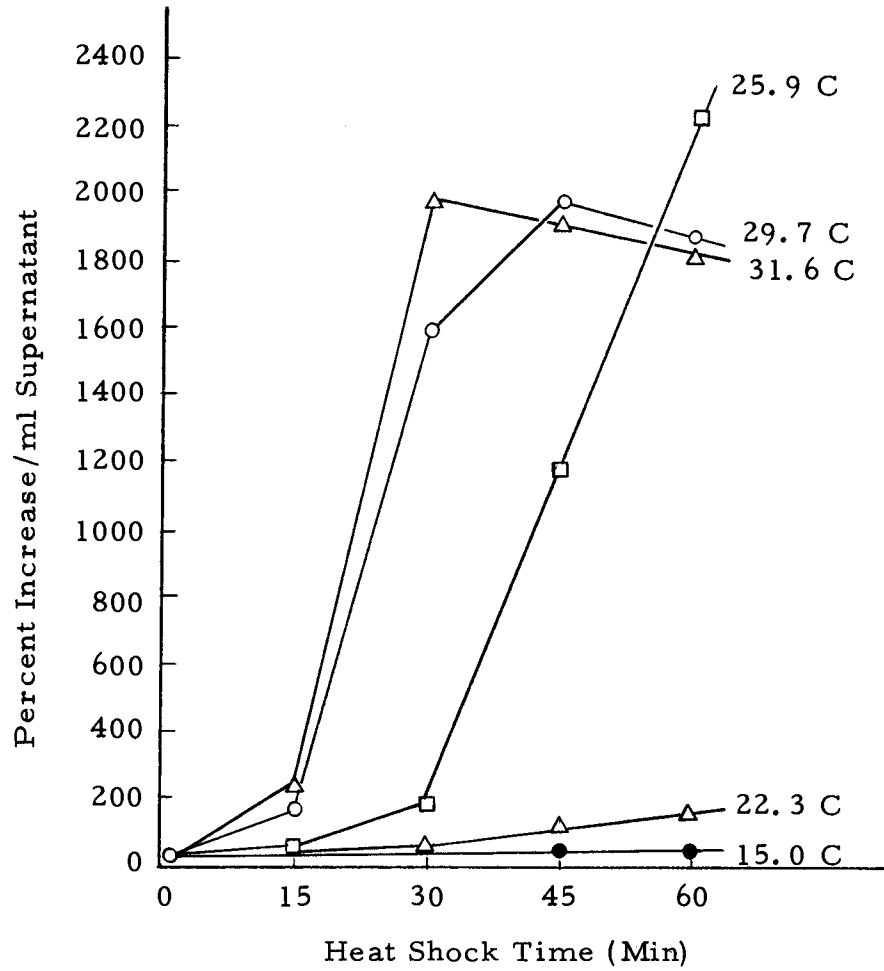

Figure 11. Heat-induced leakage of protein from Vibrio marinus MP-1. Log phase cells were washed and suspended in cold Rila sea water, salinity 27 ‰. Heating was carried out for the stated time intervals and temperatures in the polythermostat after which cell debris was removed by centrifugation at 0 C. The supernatants were decanted and stored in the refrigerator until analyzed. Protein concentration was determined by the method given in the Materials and Methods section.

TABLE IV. DISTRIBUTION OF ORCINOL-REACTING MATERIALS IN HOT AND COLD TCA EXTRACTS OF CELL-LYSATE AND HEAT-SHOCK SUPERNATANT.

Preparation	OD at 660 mμ	Micrograms RNA equiv.	Ratio (cold extract/hot extract)
TCA (5 percent)	0.00	0	-
Heat-shock supernatant			
Cold TCA extract	0.426	92	1.5
Hot TCA extract	0.275	60	
Cell-lysate*			
Cold TCA extract	0.216	46	0.4
Hot TCA extract	0.500	108	

*The TCA extractions and sample preparations were carried out according to the methods described in the Materials and Methods section. The cell lysate was diluted 50-fold prior to orcinol analysis. All values reported are on the basis of 0.5 ml original sample.

whereas at all temperatures above 22.3, protein was released in copious amounts. The fact that at 25.3 the rate was linear from 30 to 60 minutes as compared to the plateau reached between 30 to 60 minutes at higher temperatures, indicated that some protein was released between 15 and 30 minutes at both 29.7 C and 31.6 C which was later denatured or rendered insoluble by the heat treatment and was removed by the centrifugation process. The protein released by heat shock was shown to contain malic dehydrogenase and glucose-6-phosphate dehydrogenase activity as determined by spot plate tests.

Since dehydrogenase activity was present in the heat-shock supernatants, the possibility of using heat shocking as a method of

obtaining partially purified malic dehydrogenase from whole cells was investigated (Figure 12). The increased salinities were used in the hope of maintaining the released activity at a maximal level, and ammonium sulfate was tried as it had been previously shown by Langridge (16) and others (27, 28) to stabilize enzymes. The results indicated that a specific activity maximum was reached in the Rila salts menstrum, but that ammonium sulfate greatly inhibited the release of protein and of activity. The maximum specific activity obtained was 950 units per mg protein.

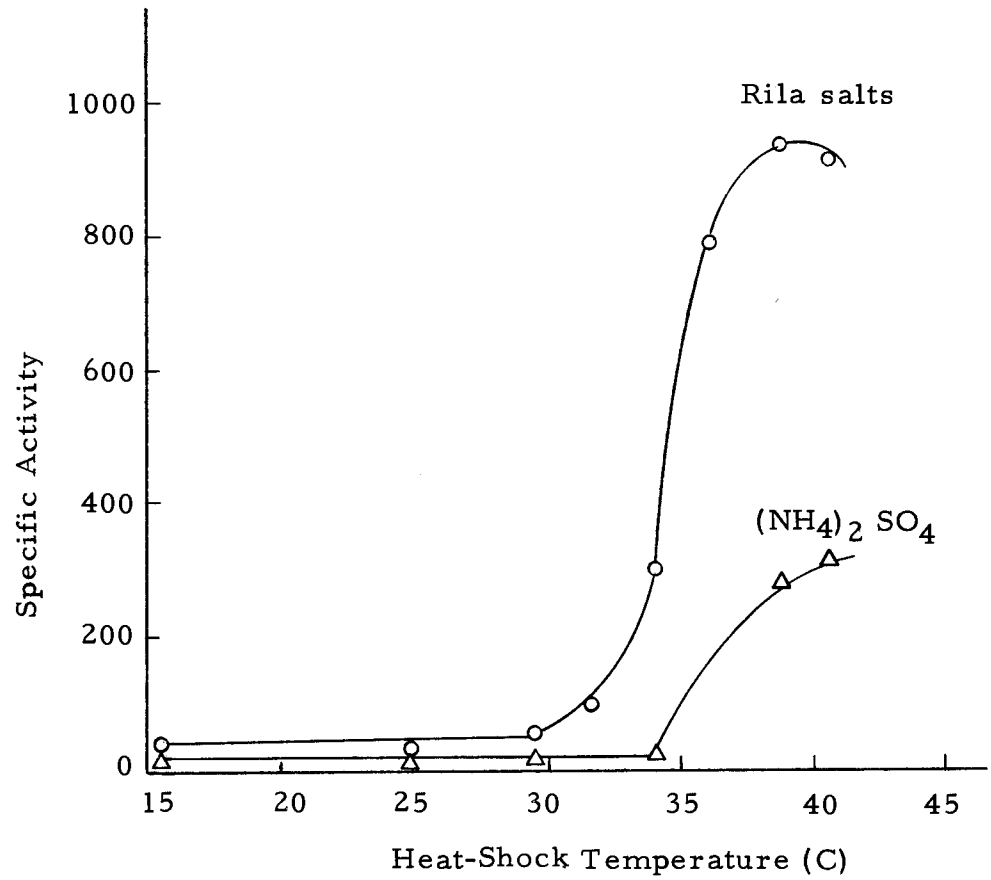

Figure 12. Specific activity of heat-shock released malic dehydrogenase from Vibrio marinus MP-1. Log phase cells were washed and suspended in cold Rila sea water, salinity 75 ‰, and cold Rila sea water - ammonium sulfate, salinity 75 ‰. Heating at the stated temperatures for 30 minutes was carried out in the polythermostat after which cell debris was removed by centrifugation at 0 C. The supernatants were decanted and stored on ice until analyzed. Protein was estimated according to the method given in the Materials and Methods section. Activity was determined by the method of Ochoa (26).

DISCUSSION

The isolation of the obligately psychrophilic marine bacterium, Vibrio marinus MP-1, was the first demonstration that psychrophilic bacteria which fit the common textbook definition exist; in the past there had been some doubt. For example, Ingraham and Stokes (1959, p. 106) stated: "Psychrophilic bacteria with an optimum temperature below 20 C, frequently described in textbooks, have rarely, if ever, been found." Many investigators in the past have described "psychrophilic" bacteria (1, 3, 13) but none of the isolates studied had a maximum growth temperature of less than 20 C. Thus, the isolation of MP-1 provided a unique opportunity to study the biochemical basis of obligate psychrophilism. The characterization of this culture microbiologically was a necessary prerequisite to biochemical studies.

Since the optimal and maximal growth temperatures for MP-1 had already been established (24), the pH range and optimum, the halotolerance, and the rate of growth remained to be determined.

Sea water has an average pH of 8.0; consequently it might be expected that the optimal pH for growth of a marine culture would be similar. This was not the case for MP-1, as the optimum pH for growth was found to be about 7.3. This organism does, however, exhibit a wide range for growth, a factor which should allow it to grow adequately in its natural environment. One might argue that nutrients

are not as prevalent in the sea as in the test tube, a factor which could effect its growth optimum; however one must consider that one bacterial function in the sea is to decompose detrital materials, an essential part of mineralization processes, and that this microenvironment may be nutrient rich (39). During the mineralization process the pH no doubt fluctuates widely, and this organism's ability to grow over a wide pH range is undoubtedly an asset in allowing it to function in concert with other bacteria present in this environment.

The commonly and long-held idea that low temperatures of incubation lead to slow growth rates is opened to question by the data shown in Figure 2. This data has been further substantiated by Morita and Albright (22) who demonstrated that MP-1, when cultured under optimum laboratory conditions, could reach a cell number of 13×10^{11} per ml. From this it may be implied that a truly psychrophilic culture is not necessarily slow growing. These data tend to refute the concept that slow growth rates, as well as low cell yields, are manifestations of low incubation temperatures for bacteria, especially obligate psychrophiles. However, when the incubation temperature was at 4 C, a temperature close to that of the natural environment, cell production was lower. These data lead one to believe that, in the natural environment, growth occurs at a slower rate than that at which the cell grows under optimal conditions. Since detrital materials settle to the bottom at varying but slow rates in the oceans, there is

no absolute requirement that bacteria grow at a rapid rate in order to serve a useful function. The fact that they grow at all at 4 C should allow them to function in the marine psychrosphere. Indeed, the obligate psychrophiles are perhaps more functional below the thermocline than facultative psychrophiles due to their functional capacity at lower temperatures.

This organism grows over a wide range of salinities as well as pH. The data obtained indicate that a large fluctuation in salinity from 15 ‰ to 50 ‰ is tolerated by MP-1. Salinity variations of this magnitude can occur in estuaries and at both poles due to melting of the polar ice caps, but are not found in the open oceans. Consequently the salinity tolerance range of MP-1 appears to be of little significance, except that it will not grow at salinities less than 10 ‰. This fits the general definition of a marine organism (18).

In the oceans a wide temperature fluctuation, especially above the thermocline, occurs which ranges from -1.8 - 28 C (39). The lower ranges (below 20 C) have already been discussed in relation to MP-1, but the upper range has not. Since MP-1 will not grow at temperatures above 20 C, one would not expect to find it in tropical surface waters, except where severe upwelling was occurring. To date, no definitive data are available on this point. However, as this upper temperature limit for growth of MP-1 is within the oceanic temperature fluctuation range, the organism's distribution, particularly

its vertical distribution within a water column, could be temperature limited. In view of this and of the implied involvement of marine psychrophiles in biological conversion processes in the oceans, the effects of temperature above 20 C on MP-1 were studied.

The data in Table II demonstrate that heating MP-1 at deleterious temperatures for varying lengths of time can render the culture nonviable, even if excess nutrient is present. In fact, a 6.25 hour treatment at 28.8 C kills the culture. This time period is quite short when one considers the kinetics of dynamic oceanic processes. Since the psychrophilic bacteria probably are not continuously subjected to a rich-nutrient environment in situ, the effect of inimical temperatures was studied when MP-1 was suspended in a nutrient-free menstrum. A striking difference was noted at 25.0 C whereas at 31.0 C nutrient appeared to have little influence on the cell's ability to survive. However at 25.0 C, when in rich nutrient, the cells remained viable whereas they lost viability rapidly if no nutrient was available. Thus, at 25.0 C, the nutrient either protected the culture from thermal damage, or it allowed the cells to rapidly repair any damage which might have occurred. This suggests that so long as the psychrophile remains below the thermocline in situ it should retain viability, whereas it can live at 20 - 25 C for at least 9.0 hours only if it is in a rich-nutrient microenvironment. This phenomenon of viability loss at relatively low temperatures appears to be a characteristic of obligate psychrophilism

as expressed by MP-1. The biochemical reasons for this viability loss were therefore investigated as they seemed important to the understanding of psychrophilism.

The exact site and nature of thermal damage to MP-1 is difficult, if not impossible, to assess. There were, at the beginning of this investigation, a number of feasible possibilities. Among these were nucleic acid degradation, nucleic acid lability, enzyme inactivation, cell membrane damage, synthesis and accumulation of toxic end-products, changes in lipid state, and loss of capability to synthesize new message (i.e., RNA and DNA synthesis), and inability to synthesize amino acids at higher temperatures. Enzyme inactivation was studied by Langridge (16) using malic dehydrogenase. It was found that this enzyme was labile at temperatures above 20 C, the precise upper temperature limit of growth of MP-1. Whether or not, on the basis of this single protein, it can be stated that enzyme lability is a definite reason for the 20 C maximum growth temperature of this organism is doubtful. Further studies on other catalytic proteins, especially those more closely associated with cell division and nucleic acid synthesis, must be obtained as corroborative evidence. However the validity of suspecting protein lability as a cause has been established.

DNA lability, one of the above cited possibilities, has been examined. It was found that purified DNA from MP-1 was not

thermolabile at a low temperature, as determined by measuring the hypochromic shift at 260 mμ; it melted at 89.0 C. This value was well within the range listed by Marmur (19) for a series of bacterial genera. This data ruled out thermolabile DNA as a feasible possibility for the maximum temperature limit of growth. This does not mean that DNA is not involved in psychrophilism. Undoubtedly the specific message governing the phenomenon of psychrophilism is contained within the DNA. This message is not so different however, that it affects the Tm value significantly.

In view of the changes in lipid saturation in E. coli (20) and Candida (14) when grown at two different temperatures, MP-1 was grown at 4 C and 15 C to determine if the lipid content would change. The result indicated that the percent extractable lipid remained constant. The result with E. coli was explained on the basis of a shift in metabolic pattern with temperature change to satisfy the cell's requirements for growth and viability maintenance at the new environmental temperature. Since MP-1 was isolated from an environment of 3.24 C (4, 24) and grows well at 15 C, it does not seem odd that the lipid content remained constant with temperature shifts within its normal functional range. E. coli normally does not have a history of low temperature exposure, a factor which could be important. It is also possible that MP-1 is not as versatile metabolically as the facultative authtroph, E. coli, and consequently changes in temperature

might only affect growth rate and not metabolic patterns. However, as the attempts to separate the extracted lipids by chromatography failed, no definitive conclusion regarding the degree of saturation could be drawn.

Low temperature seems to affect growth rate, but not metabolic patterns or the biochemical constitution of MP-1 (8, 22); there appears to be no biochemical lesion induced in this culture by low temperature incubation. However, it was noted by Robison (30) that incubation at temperatures greater than 20 C did produce lesions as evidenced by viability loss and the appearance of 260 mμ absorbing materials in cell supernatants. Therefore, the kinetics and general identification of materials released during heat shock were investigated. Kinetically, a definite correlation between time and temperature was found, i.e., heating longer at 25 C was required as compared to 31 C to release commensurate amounts of materials into the supernatant. Leakage was definitely heat-induced, as it was not found to occur at 15 C. If the temperature data in Figures 6 - 8 are cross plotted, it can be seen that leakage starts at about 20 - 22 C within 60 minutes heating time. This temperature is very near the maximum temperature of growth, and could therefore be closely related to viability loss.

The nature of the thermal lesion was originally thought to be a simple membrane rupture which allowed the soluble cellular materials

to leak out into the surrounding menstrum. To test this hypothesis, four individual components in the supernatants were analyzed: protein, DNA, RNA, and free amino acids. If the simple rupture concept was correct, the various components might have appeared in the supernatants at nearly the same time or in an order of increasing molecular weights as might be expected by simple diffusion. The data indicate that neither of these occurred. In fact DNA (larger molecular weight than protein on the average) appeared more slowly than protein, and the comparatively small molecular weight materials, the amino acids, appeared last rather than first as one would expect. It is also possible that the rates of appearance were related to internal concentration or that the lesion was of a more subtle nature than simple membrane rupture, i.e., the order of component release was under cellular control. It was suggested that the amino acid component might be of a different qualitative nature if its release was under cellular control. Analysis of amino acid preparations derived from the cells by heat shock at first glance confirmed this idea. It was found that the non-polar amino acids were released more readily at lower temperatures than at the higher ones, whereas for all other groups the opposite was true. However, another possibility existed. If one supposed that hydrophobic lipid was not released from the cells by heat shocking, the cell residues would have become more hydrophobic as other hydrophilic materials moved into the supernatant.

This change in hydrophilic-hydrophobic balance could have affected the amino acid distribution found; i. e., the polar amino acids would have been attracted to the hydrophobic environment and the hydrophilic materials to the aqueous environment. Furthermore, Silberman (34) has demonstrated that the lipids of Pseudomonas aeruginosa concentrate and bind non-polar amino acids in the intact cells. However this culture, when heat killed, lost that ability. The undamaged cells remaining in the heat-shock preparation could also be of considerable importance. Thus the amino acid picture could be manifest in either the cell rupture or membrane-control lesion hypothesis. Further study of this situation is definitely needed to prove or disprove these suggestion. The qualitative nature of the amino acids at each time-temperature condition, as well as the lipid distribution in the residue and the heat-shock supernatants, should be determined before any definite conclusions can be drawn. At this time, the nature of the control of amino acid release from MP-1 by heat-shock is unsettled.

It is apparent from the above data and argument that one site of thermal lesion is the cell membrane, and that the nature of this lesion may be more complex than simple rupture as some component order as well as qualitative amino acid make-up control is exerted. It appears quite impossible that nucleic acid compounds having very large molecular weights could get into the supernatants without passing through a membrane fissure. If polymeric nucleic acids could be

found in these supernatants, it would be a strong argument for the rupture hypothesis. Consequently studies on the polymeric nature of the RNA in heat-shock supernatants were initiated. The heat-shock condition for this study, 25 C for 60 minutes, was selected as copious amounts of orcinol-reacting material were released whereas only a small amount of diphenylamine-reacting materials (DNA origin) were found. Two analytical methods were used; Sephadex G-25 gel filtration column chromatography and hot-cold TCA extraction (5, 32). Since G-25 excludes compounds of molecular weight greater than 25,000, any 254 mμ absorbing material which could be found which was not excluded must be of a lower molecular weight, or therefore partially degraded RNA. The elution patterns shown in Figure 10 indicated that both polymeric and non-polymeric materials were present in cell lysates and in 25 C-60 minute heat-shock supernatants. Hot and cold TCA extractions provided the data in Table IV. These data indicated that indeed the majority of orcinol-reacting material in the heat-shock supernatant was of a smaller molecular weight. The opposite was true for cell lysate. From these data it can be concluded that a small amount of polymeric RNA was released from the cells by heating, but the majority was of molecular weight smaller than 25,000. Whether or not degradation occurred prior to, or during, heating is not clear; however if it occurred first and the degradation products could leave the cell, no evidence was found by

Sephadex column analysis (supernatant from unheated cells) in support. It is possible that heating induced degradation of RNA inside the cell which preceded leakage, or that the RNA was hydrolyzed enzymatically after leaving the cell. The fact that some polymeric RNA was released by heat shock argues against the controlled heat-induced leakage (subtle membrane lesion) hypothesis; indeed it argues for cell rupture. It is possible also that RNA is bound to the cell membrane, most likely internally. Even so the polymeric material would still have to pass through a fissure to get outside the cell.

The analysis of protein in the heat-shock supernatants was crucial for deciding between the rupture and permeability lesion hypotheses. If the protein was not protein at all but polypeptides of smaller molecular weight (Biuret reacting), the rupture hypothesis could be strongly argued against, unless excessive proteolysis was occurring during heat-shocking. Three facts about this protein imply that it was intact and not degraded: it was TCA insoluble, it contained catalytic activity, and it reached a maximum concentration if the temperature of heat-shocking was high. In the latter case it appeared that a portion of the protein was heat labile, i. e., it became insoluble during heat-shocking, and was removed with the cell debris during centrifugation (Figure 11). Figure 12 shows that malic dehydrogenase activity was released by heat-shocking. The specific activity reached a maximum and was quite high as compared to that found in crude lysates

(16). Therefore heat-shocking appears to be a method of obtaining a partially purified malic dehydrogenase from MP-1. This activity no doubt represents a relatively heat-stable fraction. The fact that 7.5 percent ammonium sulfate delays the release of protein into the supernatant indicates that the membrane is in some way stabilized against damage by heat. Since ammonium sulfate is known to increase the stability of some proteins (27, 28) it seems likely that the proteolipid membrane could be affected similarly.

In conclusion, it is still difficult to say definitely what the nature of the membrane lesion might be. The facts and arguments given above seem to lead to confidence in the simple rupture or fissure hypothesis.

SUMMARY

A number of observations of MP-1, both microbiological and biochemical, have been made.

Microbiologically this culture grows optimally at pH 7.3, and adequately from pH 6.7 - 8.6; it has a wide halotolerance for growth; grows maximally and rapidly at 15-16 C; will not grow at temperatures above 20.0 C; and thermal damage appears to either be inhibited or repaired in the presence of nutrients.

Biochemically, DNA is thermostable; heat-shocking induces cell damage, one form of which is a membrane lesion; polymeric and degraded RNA, DNA (diphenylamine - reacting material), catalytically active protein, and amino acids are released from the cells by heat shocking; growth temperature does not affect the percent extractable lipid in the cells; the qualitative constitution of the amino acids released at different times and temperatures is different; and heat shocking of cells may be used as a method of obtaining a partially purified preparation of malic dehydrogenase from MP-1.

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APPENDIX

APPENDIX - EQUIPMENT

Name of Instrument	Cited in Thesis as	Source
Bausch and Lomb Spectronic 20 Spectrophotometer	spectrophotometer	Bausch and Lomb, Inc. Rochester, New York
New Brunswick Psychro-Therm Controlled Environment Incubator-Shaker	PsychroTherm	New Brunswick Scientific Co. New Brunswick, New Jersey
Gradient Temperature Incubator	Polythermostat	Home constructed
Ivan Sorvall Superspeed RC-2 Refrigerated Centrifuge	RC-2	Ivan Sorvall, Inc. Norwalk, Conn.
Ivan Sorvall Szent-Gyorgyi and Blum Continuous Flow System	Continuous flow	Ivan Sorvall, Inc. Norwalk, Conn.
Beckman Model DU Spectrophotometer	DU	Beckman Instruments Co. Fullerton, California
Aminco Refrigerated Water Bath	Water bath	American Instruments Co., Inc. Silver Springs, Maryland
VirTis Lyophilizer	Lyophilizer	VirTis Research Equipment, Gardiner, New York
Beckman Automatic Amino Acid Analyzer	Amino acid analyzer	Beckman Instruments Co. Fullerton, California

Equipment (cont.)

Name of Instrument	Cited in Thesis as	Source
Pharmacia Sephadex Column 1" x 18"	Pharmacia column	Pharmacia Uppsala, Sweden
ISCO Model UA Recording Ultraviolet Analyzer	ISCO monitor	Instrument Specialties Co. Lincoln, Nebraska
Raytheon 10 kc Sonic Oscillator	Sonorator	Raytheon Manufacturing Co. Waltham, Mass.

APPENDIX - CHEMICAL

This appendix lists those chemicals and their sources which are considered to be unusual or not commonly stocked in the laboratory.

Substance	Source
L-alanine	Nutritional Biochemicals Corp. Cleveland, Ohio
Bovine serum albumin	Sigma Chemical Co. St. Louis, Missouri
Desoxyribonucleic acid (DNA) Salmon sperm, type III	Sigma Chemical Co. St. Louis, Missouri
Dextran 2000	Pharmacia Uppsala, Sweden
Diphenylamine	J. T. Baker Chemical Co. Phillipsburg, New Jersey
Dow Corning Anti-foam A Silicone Spray	Dow Corning Corp. Midland, Michigan
Ethylenediaminetetraacetic acid (EDTA)	Sigma Chemical Co. St. Louis, Missouri
Nicotinamide adenine dinucleotide, reduced (NADH)	Calbiochem Los Angeles, California
Ninhydrin (1, 2, 3-triketohydrindene)	Eastman Organic Chemicals Rochester, New York
Orcinol	Sigma Chemical Co. St. Louis, Missouri
Oxalacetic acid (OAA)	Nutritional Biochemicals Corp. Cleveland, Ohio
Polypeptone	Difco Laboratories Detroit, Michigan

Chemical (cont.)

Substance	Source
Proteose peptone	Difco Laboratories Detroit, Michigan
Ribonuclease (RNase) bovine pancreas, crystalline	Sigma Chemical Co. St. Louis, Missouri
Ribonucleic acid (RNA) yeast	Sigma Chemical Co. St. Louis, Missouri
Rila marine salts	Rila Products, Co. Teaneck, New Jersey
Sephadex G-25 fine, bead form	Pharmacia Uppsala, Sweden
Sodium lauryl sulfate	Sigma Chemical Co. St. Louis, Missouri
Succinic acid	J. T. Baker Chemical Co. Phillipsburg, New Jersey
Tris (hydroxymethyl) aminomethane (Tris)	Sigma Chemical Co. St. Louis, Missouri
Tween 80	Scientific Supplies Co. Seattle, Washington
Yeast extract	Difco Laboratories Detroit, Michigan

APPENDIX - REAGENTS AND SOLUTIONS

This appendix gives, in alphabetical order, the procedures for the preparation of all reagents, solutions, and their components.

Acetaldehyde, aqueous; 16 mg per ml

Weigh a known amount of pure acetaldehyde and dilute with distilled water such that acetaldehyde is 16 mg per ml final concentration, i.e., the final volume is the weight in mg of acetaldehyde divided by 16. Use in the preparation of the diphenylamine reagent.

Acetate - EDTA

Dissolve 246 g sodium acetate and 0.290 g ethylenediaminetetraacetic acid in 800 ml distilled water. Adjust the pH to 7.0 and dilute to a final volume of 1,000 ml with distilled water.

L-alanine; 0.5 micromoles per ml

Dissolve 4.45 mg L-alanine in 100 ml distilled water. Use as the standard solution for the ninhydrin assay for amino acids.

Ammonium sulfate - Rila salts (ARS); 75 ‰

Dissolve 73.0 g ammonium sulfate and 0.2 g Rila salts in 800 ml distilled water. Adjust the pH to 7.3 and dilute to a final volume of 1,000 ml with distilled water.

Biuret reagent

Add 250 ml aqueous 0.6 percent copper sulfate solution to an equal volume of aqueous 2.4 percent sodium potassium tartrate solution and mix thoroughly. Carefully add 300 ml aqueous ten percent sodium hydroxide with continuous stirring, and dilute to 1,000 ml with distilled water. For stability, dissolve a few crystals of potassium iodide in the reagent. Store in the dark. Use for the protein assay.

Bovine serum albumin (BSA); 10.0 mg per ml

Dissolve 100 mg BSA in 10.0 ml distilled water. Use as the standard protein solution in the protein assay with Biuret reagent.

Chloroform - isoamylalcohol (CI)

Mix 240 ml chloroform with 10.0 ml isoamyl alcohol.

Chloroform - methanol (CM)

Mix 200 ml chloroform with 100 ml methanol.

Reagents and Solutions (cont.)

Citrate buffer

Dissolve 21.008 g citric acid in 200 ml aqueous 1 N sodium hydroxide solution and dilute to a final volume of 500 ml with distilled water. Use in the preparation of the ninhydrin reagent.

Desoxyribonucleic acid (DNA)

For use as the standard DNA solution (100 micrograms per ml) in the diphenylamine assay for DNA, dissolve 10.0 mg salmon sperm DNA in 100 ml 0.5 N perchloric acid. If necessary, heat in the steamer to completely solubilize the DNA. Do not use this preparation for Sephadex chromatography as DNA is degraded by the acid treatment.

To prepare DNA for Sephadex chromatography and melting point studies, dissolve 5.0 mg in 100 ml dilute saline-citrate or distilled water. Do not heat. This preparation should be polymeric.

Dextran 2000; 0.1 percent

Dissolve 10.0 mg Dextran 2000 in 10.0 ml distilled water. The solution is deep blue in color.

Diluent solution

Mix 300 ml n-propanol with 300 ml distilled water. This solution is used in the ninhydrin assay for amino acids.

Diphenylamine stock solution

Dissolve 1.0 g recrystallized diphenylamine in 100 ml glacial acetic acid; add 1.5 ml concentrated sulfuric acid; mix thoroughly. Store in the dark.

Diphenylamine reagent

Mix 20 ml diphenylamine stock solution with 0.1 ml acetaldehyde (aqueous) solution. Use immediately after preparation in the diphenylamine assay for DNA.

Ninhydrin reagent

Dissolve 0.80 g stannous chloride in 500 ml citrate buffer. Mix thoroughly with 500 ml pure methylcellosolve containing 20 g ninhydrin. Prepare fresh daily. Use in the assay for amino acids.

Nicotinamide adenine dinucleotide, reduced (NADH); 0.002 M

Dissolve 14.7 mg NADH· $2H_2O$ disodium salt in 10.0 distilled water.

Reagents and Solutions (cont.)

Orcinol reagent

Dissolve 1.0 g recrystallized orcinol and 0.5 g ferric chloride in 100 ml 7.25 N hydrochloric acid. Use immediately in the assay for RNA.

Oxalacetic acid (OAA); 0.008 M

Dissolve 10.46 mg OAA in 8.0 ml distilled water. Adjust the pH to about 7.4 and dilute to a final volume of 10.0 ml. Prepare fresh daily.

Pure-solvents-upper-phase (PSUP)

Thoroughly shake together 80 ml chloroform, 40 ml methanol, and 30 ml distilled water. Allow the phases to separate by standing at room temperature. The upper phase is PSUP.

Ribonuclease (RNase); 0.2 percent

Dissolve 20 mg crystalline RNase in 10.0 ml 0.15 M sodium chloride at pH 5.0. Heat this solution at 80 C for ten minutes to inactivate any contaminating DNase.

Ribonucleic acid (RNA)

For use as the standard RNA solution (100 micrograms per ml) in the orcinol assay for RNA, dissolve 10.0 mg yeast RNA in 100 ml 0.5 N sodium hydroxide. If necessary, heat in the steamer to completely solubilize the RNA. Do not use this preparation for Sephadex chromatography as RNA is degraded by the alkaline treatment.

To prepare RNA for Sephadex chromatography, dissolve 5.0 mg in 100 ml dilute saline-citrate (DSC) or in distilled water. If a residue remains, filter the solution through a thoroughly washed Whatman Number One filter paper. Do not heat. This preparation should be polymeric.

Rila sea water (RSW)

Dissolve 35.0 g Rila salts in 800 ml distilled water, adjust the pH to 7.3, and dilute to 1,000 ml with distilled water to get one liter 35 ‰ RSW. For 27 ‰, dissolve 27.0 g Rila salts in 800 ml distilled water, adjust the pH to 7.3, and dilute to 1,000 ml with distilled water.

Reagents and Solutions (cont.)

Saline-citrate, dilute (DSC)

Dissolve 0.877 g sodium chloride and 0.387 g trisodium citrate in 800 ml distilled water, adjust the pH to 7.0 ± 0.2, and dilute to 1,000 ml with distilled water.

Saline-citrate, normal (NSC)

Dissolve 8.77 g sodium chloride and 3.87 g trisodium citrate in 800 ml distilled water, adjust the pH to 7.0 ± 0.2, and dilute to 1,000 ml with distilled water.

Saline-citrate, concentrated (CSC)

Dissolve 87.7 g sodium chloride and 38.7 g trisodium citrate in 800 ml distilled water, adjust the pH to 7.0 ± 0.2, and dilute to 1,000 ml with distilled water.

Saline-EDTA

Mix 400 ml aqueous 0.15 M sodium chloride and 400 ml aqueous 0.1 M ethylenediaminetetraacetic acid, adjust the pH to 8.0, and dilute to 1,000 ml with distilled water.

Sodium chloride, 0.73 percent

Dissolve 0.73 g sodium chloride in 100 ml distilled water.

Sodium lauryl sulfate, 25 percent

Dissolve 25 g sodium lauryl sulfate in 100 ml distilled water. The solution may remain slightly turbid.

Sodium perchlorate, 5 M

Dissolve 61.25 g sodium perchlorate in 100 ml distilled water.

Trichloroacetic acid (TCA)

Dissolve 5.0 g TCA in 100 ml distilled water for a five percent solution, or 10.0 g TCA in 100 ml distilled water for a ten percent solution.

Tris (hydroxymethyl) aminomethane-sulfate buffer (Tris-sulfate); 0.2 M

Dissolve 24.2 g Tris buffer salt in 800 ml distilled water, adjust the pH to 7.4 with sulfuric acid, and dilute to a final volume of 1,000 ml with distilled water.