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Title: TEMPERATURE-SALINITY EFFECTS ON NET PROTEIN SYNTHESIS AND VIABILITY OF VIBRIO MARINUS MP-1, AN OBLIGATELY PSYCHROPHILIC MARINE BACTERIUM

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Abstract approved: Richard Y. Morita

The relationship of temperature and salinity to protein synthesis was determined for cells of Vibrio marinus, MP-1. Protein synthesis was measured by the incorporation of radioactive proline into hot trichloroacetic acid precipitable material. At all salinities protein synthesis occurred at 15°C and 20°C but not at 25°C. The critical temperature of the lesion in protein synthesis increased with increasing salinity of the growth medium. A significant inhibition of protein synthesis occurred at 22°C at a salinity of 25‰, but no inhibition of protein synthesis occurred at a salinity of 35‰ until the cells were incubated for 20 minutes at 24°C. The possibility that the thermal lesion involved precursor accumulation mechanisms rather than protein synthesis at salinities between 25‰ and 35‰ was eliminated by determining the uptake of labeled proline by whole cells. At 40‰,

the uptake of extracellular amino acids was inhibited at 24°C and preceeded the inhibition of precursor into protein.

RNA synthesis studies were determined at incubation temperatures inhibitory to protein synthesis. RNA synthesis was measured by the incorporation of radioactive uracil into cold trichloroacetic acid precipitable material. Total RNA synthesis continued for 50 minutes at 22°C in growth media at salinities between 15‰ and 35‰. At a salinity of 40‰, incorporation of uracil into RNA decreased after 20 minutes of cell incubation at 22°C. At growth medium salinities between 15‰ and 30‰, total RNA synthesis continued at 15°C and 22°C, but cellular protein synthesis was inhibited by either temperature or salinity effects.

Studies of cell viability loss at 22°C and 25°C in growth media at salinities of 25‰ and 35‰ showed that the onset of cell death occurs simultaneously with thermal inhibition of protein synthesis. The death of cells occurs more rapidly as the salinity of the growth medium is lowered and as the temperature of cell incubation is increased.

Temperature-Salinity Effects on Net Protein Synthesis and Viability of Vibrio marinus MP-1, an Obligately Psychrophilic Marine Bacterium

by

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Typed by Sammy McMurphey for Mary Faith Cooper

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INTRODUCTION

The oceanic factors of temperature and salinity vary in short periods of time and space in near-shore environments. If these two environmental parameters prohibit the growth of marine bacteria, a lower rate of metabolic degradation of organic materials suspended in sea water may result.

Growth studies on the obligate marine psychrophile Vibrio marinus MP-1 indicate that the salinity of the growth medium affects the maximum temperature for cell viability (74). The highest maximum growth temperature, 20°C, occurs in medium at a salinity of 35‰, and the maximum growth temperature is lowered to 18°C at salinities above 37‰ and below 23‰. In the sequence of events in cells exposed to restrictive growth temperatures, the loss of cell viability preceeds the leakage of cellular constituents and the subsequent lysis of cells (37). Log phase cells are more sensitive to thermally induced viability loss than are stationary phase cells. This suggests physiological processes active during rapid cell growth as possible areas of thermal damage. Cells suspended in a complex medium continue to synthesize RNA three hours after exposure to a temperature 5°C above the maximum growth temperature

of 20°C. However, protein and DNA synthesis stop within one hour after the temperature increase (54). The medium used in the experiments of Morita and Albright (54) does not allow rigid control of, or variations in the salinity of, the growth medium. Thus correlation of the maximum temperature for growth with the thermal inhibition of protein synthesis, as affected by the salinity of the growth medium, is not possible. This thesis describes experiments designed to determine if the salinity of the growth medium implicates factors involved in the translation of protein or in the transcription of RNA in cellular thermal damage at temperatures immediately above the maximum for growth.

LITERATURE REVIEW

Since 90 per cent of the marine environment is below 5°C (83), we may predict the selection of bacteria specifically adapted to grow as well in the cold ocean as mesophiles and facultative psychrophiles grow in warmer environments. The thermal sensitivity of bacteria in the ocean was investigated in 1940 (84), but the existance of obligate psychrophiles was still in doubt seven years ago (32).

In 1964 Morita and Haight (57) reported the isolation of a marine bacterium, classified as Vibrio marinus (7), which fit Stokes' definition of a psychrophile: a bacterium which develops easily visible growth within about one week at 0°C (76). The optimum and maximum temperatures for growth of an obligately psychrophilic strain of V. marinus, MP-1, in complex medium are 15°C and 20°C, respectively (57). The growth response of V. marinus MP-1 at low temperature differs from that of a facultatively psychrophilic strain of Pseudomonas. Growth of the obligate psychrophile at low temperatures yields high cell numbers in 24 hours, whereas the facultative psychrophile tolerates the low growth temperature, but the cell yield after 24 hours is low (55). Previous investigations have implicated various mechanisms in the physiology of thermal death of psychrophiles. Investigations of thermosensitive enzymes of psychrophiles are numerous (6, 10, 13, 21, 41, 48, 50, 51, 56, 65, 69, 71, 75, 77). Rose (69) predicts that inhibition of one or more

enzymes involved in glucose metabolism may result in the accumulation of intermediates which act as toxic materials by inhibiting the activity of other enzymes.

Other studies have demonstrated that leakage of cellular constituents and cell wall and/or membrane damage are correlated with thermal death of psychrophiles (4, 14, 22, 24, 33, 59). In many of these investigations, the sequence of events involved in thermal death was not determined. For example, until Kenis and Morita (37) demonstrated that the loss of cell viability preceeds leakage and subsequent cell lysis of V. marinus MP-1 at temperatures immediately above the maximum for growth, it was not known if leakage and membrane damage result in or result from thermal death of the cell. Several investigators have recognized the importance of differences in the lipid content of cell walls of psychrophilic and mesophilic organisms grown at low temperatures (35, 36, 49, 68, 70). These studies indicate that among the adaptations of organisms to growth at low temperatures is an increase in the proportion of unsaturated fatty acids with decreasing growth temperature. The lower melting point of the unsaturated fatty acids may allow a lower temperature of efficient function of the cell membrane, an area of high cellular lipid content.

The destruction of RNA has been demonstrated in thermal injury to Staphlococcus aureus (72). In another investigation, an RNAse

active at moderate temperatures above the maximum for growth was inactivated at higher incubation temperatures (3). Although leakage of RNA fragments has been reported among the effects of heating cells above the maximum growth temperature of V. marinus MP-1 (25), the breakdown of RNA has not been implicated as a cause of thermal death in this or in other psychrophiles. To the contrary, Krajewska (39) has shown that ribosomes from a psychrophilic Pseudomonas species are capable of functioning in a heterologous system 15°C above the maximum growth temperature of the organism. Studies by Pace and Campbell (62) correlate the maximum growth temperature of various thermophilic, mesophilic, and psychrophilic microorganisms with the thermal stability of their ribosomes, but thermal disruption of ribosomes, as determined by hyperchromicity at 260 mμ, did not occur in V. marinus MP-1 until 69°C, a temperature almost 50°C above the maximum growth temperature.

A number of earlier investigations (23, 46, 60, 81, 82) suggested the recent discoveries of thermosensitive enzymes involved in protein synthesis of obligately psychrophilic microorganisms (26, 48, 54, 71, 76). The series of papers by Malcolm (46, 47, 48) report a systematic investigation of the thermal death events in an obligate psychrophile. The direct relationship between thermal inhibition of protein synthesis and the loss of cell viability led to the discovery of three species of thermosensitive aminoacyl t-RNA

synthetase enzymes and one species of thermosensitive t-RNA. The experiments show that thermally induced changes in t-RNA are reversible, but the synthetase enzymes are irreversibly altered by heat.

Other investigators have used temperature sensitive mutants of various organisms for genetic studies and to isolate specific areas of the macromolecule synthesizing systems (11, 12, 28, 29, 30). Studies using temperature sensitive mutants are extensively discussed in a recent review (15). Other authors have reviewed the physiological aspects of thermal death of psychrophiles discussed above (14, 16, 17, 34, 52, 53, 75, 83).

Harder and Veldkamp (26) investigated an obligately psychrophilic Pseudomonas species grown at different temperatures in continuous culture. They related differences in the amount of cellular protein and RNA synthesized in log phase cells to variations from the optimal temperatures for growth and respiration of the organism. Cells grown at temperatures other than the optimum for growth synthesize more RNA to increase the concentration of cellular respiratory enzymes. The greater amount of RNA synthesized thereby compensates for either impaired protein synthesis or enzyme function at temperatures above the optimum for growth and for the decrease in reaction rate of the enzymes at the lower temperatures. Marr and Ingraham (49) have shown that high temperatures of incuba-

tion of Escherichia coli prevent the repression of enzyme synthesis. Rose (69) also suggested that the induction and repression of enzymes in psychrophiles may be thermosensitive.

Effects similar to those reported in V. marinus MP-1 (74), indicating an increase in the maximum temperature for growth by increasing the concentration of NaCl in the growth medium, have been reported elsewhere (20, 42). Both K^+ and Na^+ are specifically required by marine bacteria for cell growth and for oxidation of exogenous substrates (7, 43, 63, 79). A number of investigators have suggested that Na^+ functions in the uptake of exogenous substrates while K^+ is required for intracellular substrate oxidation (8, 9, 45). Since the amount of Na^+ required for uptake varies with the substrate, these differences are explained by postulating the presence of a number of permeases in the cell membrane requiring different Na^+ concentrations for activation. Payne (63, 64) suggested that Na^+ is involved in the induction of permease enzymes of marine bacteria, however Drapeau (8, 9) demonstrated that Na^+ dependent accumulation of a non-metabolite occurred in the presence of chloramphenicol, an inhibitor of enzyme synthesis. From these studies, the function of Na^+ in the oxidation of endogenous substrates appears to be intracellular (44) with the capacity to activate enzymes previously induced. Likewise, studies by Miller demonstrated that an increase in the concentration of Na^+ up to 0.7 m stimulated the rate

of enzyme activity of glucose-6-phosphate dehydrogenase obtained from V. marinus MP-1 (51). The presence of NaCl activated this thermosensitive enzyme after two minutes exposure to 44°C, a temperature normally inactivating the salt-free enzyme.

MATERIALS AND METHODS

Media

SDB agar medium was composed of polypeptone (BBL), 5.0 g; yeast extract (Difco), 3.0 g; Rila Marine Mix (Rila Products, Teaneck, N. J.), 5.0 g; sodium chloride, 15.0 g; glucose, 0.5 g; succinic acid, 0.2 g; ferrous sulfate, 0.01 g; Bacto-agar (Difco), 15.0 g; distilled water, 1 liter. The pH was adjusted to 7.5 with NaOH.

Succinate salts medium (SSPU) consisted of succinic acid, 2.5 g; ammonium sulfate, 2.0 g; K_2HPO_4, 4.0 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; vitamin solution, 1 ml; trace elements, 1.0 ml; proline, 10 mg; uracil, 10 mg; NaCl was added in the following concentrations for each salinity: 15 parts per thousand (‰), 8.5 g; 20‰, 13.7 g; 25‰, 18.8 g; 30‰, 24.0 g; 35‰, 29.2 g; 40‰, 34.5 g. The total volume of each medium was adjusted to 1000 ml with distilled water. The pH was adjusted to 7.8 with NaOH. The vitamin solution contained nicotinamide, 400 mg; thiamine, 100 mg; pyridoxine HCl, 100 mg; riboflavine, 25 mg; biotin, 1 mg; calcium-d-pantothenate, 100 mg; and distilled water to a volume of 1000 ml. The trace element solution contained the following reagent-grade chemicals: $CaCl_2 \cdot 2H_2O$, 200 mg; $FeCl_3 \cdot 6H_2O$, 100 mg; KBr, 100 mg; KI, 10 mg; $MnCl_2 \cdot 4H_2O$, 100 mg; $SrCl_2 \cdot 6H_2O$, 100 mg; H_3BO_3, 100 mg; $CoCl_2 \cdot 6H_2O$, 100 mg; and distilled water to a

volume of 100 ml.

The pH of all media was 7.4 after sterilization by autoclaving at 15 psi for 15 minutes.

Organism

The obligate marine psychrophile, Vibrio marinus MP-1, was isolated by Morita and Haight (57) in the North Pacific at a water temperature of 3.24°C and classified by Colwell and Morita (7). Monthly transfers of the stock culture were inoculated onto SDB agar slants and incubated at 5°C. New working stock cultures were established every two weeks by loop transfer from the SDB agar stock culture into 50 ml SSPU (35‰) medium in a 250 ml Erlenmeyer flask. Incubation was at 15°C in a psychrotherm incubator (New Brunswick Scientific Company) with the reciprocal shaker set at 100 strokes per minute. This working stock culture was maintained by a 10 per cent inoculum (v/v) into fresh SSPU (35‰) medium every three days.

Growth of the Culture

Five ml of the working stock culture were inoculated into 50 ml SSPU medium in a 250 ml Erlenmeyer flask and incubated in the psychrotherm for 30 hours at 15°C. Ten ml of this culture were inoculated into 100 ml of fresh medium in a 500 ml side-arm flask and incubated in the psychrotherm. Growth curves were determined on

the latter culture in SSPU media at salinities of 25‰ and 35‰. At intervals the OD was estimated at 425 mμ in a colorimeter (Bausch and Lomb Spectronic 20) with a medium blank as a standard.

Preparation of Log Phase Cell Suspension

Fifty ml of a 12 hour culture of log phase cells in SSPU medium were filtered in the cold at 4-7°C onto a sterile Millipore filter (47 mm, 0.45 μ) previously rinsed with sterile SSPU medium. Filtration was facilitated by vacuum with the negative pressure not exceeding 4 inches of Hg. The filtered cells were washed with five ml SSPU medium at 4-7°C and resuspended in fresh SSPU to an OD of 0.125 estimated at 425 mμ on a Beckman DB spectrophotometer in a quartz cuvette with a 1 cm path length against a distilled water blank. For cell preparations at salinities of 25‰ to 40‰, 12 hour log phase cultures were filtered, rinsed, and resuspended in SSPU medium at the same salinity. To obtain sufficient cells for studies run at 15‰ and 20‰, cultures were grown in SSPU medium at a salinity of 25‰. The 12 hour cultures were washed and resuspended with SSPU media at the lower salinities.

Cell Count and Corresponding Optical Density of Resuspensions at Different Salinities

The OD of log phase cell suspensions at salinities of 25‰, 30‰, 35‰ or 40‰ was compared to direct cell counts of the resuspensions.

A log phase cell suspension was prepared as described above, except that cells were resuspended in SSPU medium at three OD's ranging from 0.11 to 0.18 estimated at 425 mμ on a Beckman DB spectrophotometer with a distilled water blank. Three cell counts of each suspension were made with a Petroff-Hausser counting chamber, and the mean of the counts was computed. The OD of cells grown in SSPU media at four different salinities was compared with cell counts of log phase cell suspensions to determine if the OD of cells grown at different salinities varies appreciably.

Temperature-Viability Studies

Seven ml of a log phase cell suspension were pipetted into sterile test tubes equilibrated previously to 15°C, 22°C, and 25°C in a polythermostat constructed by Morita and Haight (57). At 30 or 60 minute intervals samples removed from the cell suspensions were serially diluted, and 0.1 ml of the diluted suspension was spread on SDB agar at 15°C. Plates containing 30 to 300 colonies were counted after 48 hours of incubation at 15°C. The mean cell count was computed to determine viable count for each sample. Studies were run for three and five hours in SSPU media at salinities of 25‰ and 35‰, respectively.

Protein Synthesis Studies

L-proline UL-C-14 (200 mC/mM; International Chemical and Nuclear Corporation) was added to a 15°C log phase cell suspension in a 125 ml Erlenmeyer flask to an activity of 0.05 μc/ml. After warming the cell suspension to each experimental temperature, four to eight ml aliquots were pipetted into test tubes temperature equilibrated in the polythermostat. Incorporation of labeled proline into protein was determined by a modification of the method described by Kennell (38). At 10 to 15 minute intervals 0.5 or 1.0 ml of cell suspension was pipetted into an equal volume of trichloroacetic acid (20% w/v) in a 1.6 x 15 mm test tube placed in crushed ice. The sample size was constant throughout each experiment. The contents of the tube were mixed thoroughly, and cold trichloroacetic acid (5%) was added to bring the total volume to three ml. This mixture was kept on ice for 30 minutes. Each tube was covered with a marble and placed in a water bath at 82°C for exactly 30 minutes. The tubes were cooled and the hot acid insoluble material was recovered by filtering the mixture through a 22 mm microfiber glass disc prefilter (AP20, Millipore) previously soaked with trichloroacetic acid (10%). The filtration pressure was set at -10 inches of Hg. Suspension tubes were rinsed twice with four ml cold trichloroacetic acid (10%), and the rinse material was filtered through the glass prefilter. The prefilter was rinsed once with four ml cold trichloroacetic acid (10%)

and twice with five ml cold ethanol (70% v/v). The filters were placed in counting vials, dried under a heat-lamp for two hours, and covered with five ml scintillation fluor solution composed of 2, 5-diphenyloxazole (PPO), 4.0 g; dimethyl 1, 4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 0.30 g; toluene, 1000 ml. Radioactivity was measured in a liquid scintillation spectrometer (Tri-Carb model 300, Packard Instrument Company). A ^{14}C-proline sample was used to determine the optimal instrument settings. A glass prefilter rinsed as a sample was used to determine background.

Uptake Studies

The uptake of ^{14}C-proline by whole cells used in the protein synthesis studies was determined by filtering 0.5 ml of cell suspension on a 25 mm cellulose membrane filter (0.45 μ; Millipore) presoaked with cold 75 per cent sea water containing 26.3 g Rila Marine Mix in 1000 ml distilled water. The filtered cells were immediately rinsed with 2.0 ml cold 75 per cent sea water. The filters were placed in counting vials, dried and covered with five ml scintillation fluor solution. Uptake of ^{14}C-proline was estimated by counting the emission of radioactivity as described above in protein synthesis studies.

Ribonucleic Acid Synthesis Studies

Uracil UL-H-3 (5.61 c/mM; New England Nuclear Corporation) was added to a 15°C log phase suspension of cells to an activity of 1 μc/ml. Samples were treated as described above for protein synthesis except that trichloroacetic acid (10%) was used instead of trichloroacetic acid (5%). The 30-minute incubation at 82°C, used to resolubilize RNA, was eliminated. The appropriate settings for the scintillation spectrometer were determined using a sample prepared for RNA analysis.

RESULTS AND DISCUSSION

Preliminary investigations established constant experimental conditions to determine the effects of the variable parameters of temperature and salinity. The only constituent which varied in concentration in the synthetic SSPU media at the six salinities was NaCl. Media of defined composition allowed a more precise calculation of the oceanographic parameter of total salinity than if complex media containing large quantities of organic material were used. The use of succinic acid rather than glucose eliminated the necessity for separate autoclaving and subsequent mixing of carbon source and salts of the media. Unlabeled l-proline and uracil were added to SSPU media to insure the activation of cellular accumulation systems, if present in Vibrio marinus MP-1, prior to the introduction of radiotracer compounds. This alleviated an initial time lag in the uptake and incorporation of labeled precursors into whole cells and cellular macromolecules, respectively.

All cell suspensions studied at the six salinities were obtained from the middle of the log phase growth. Growth curves in media at salinities of 25‰ and 35‰ were similar, with the cultures entering log phase growth approximately four hours after inoculation and with inflection points, indicating the end of exponential growth, at 21 hours (Fig. 1).

The size and morphology of cells grown at different salinities

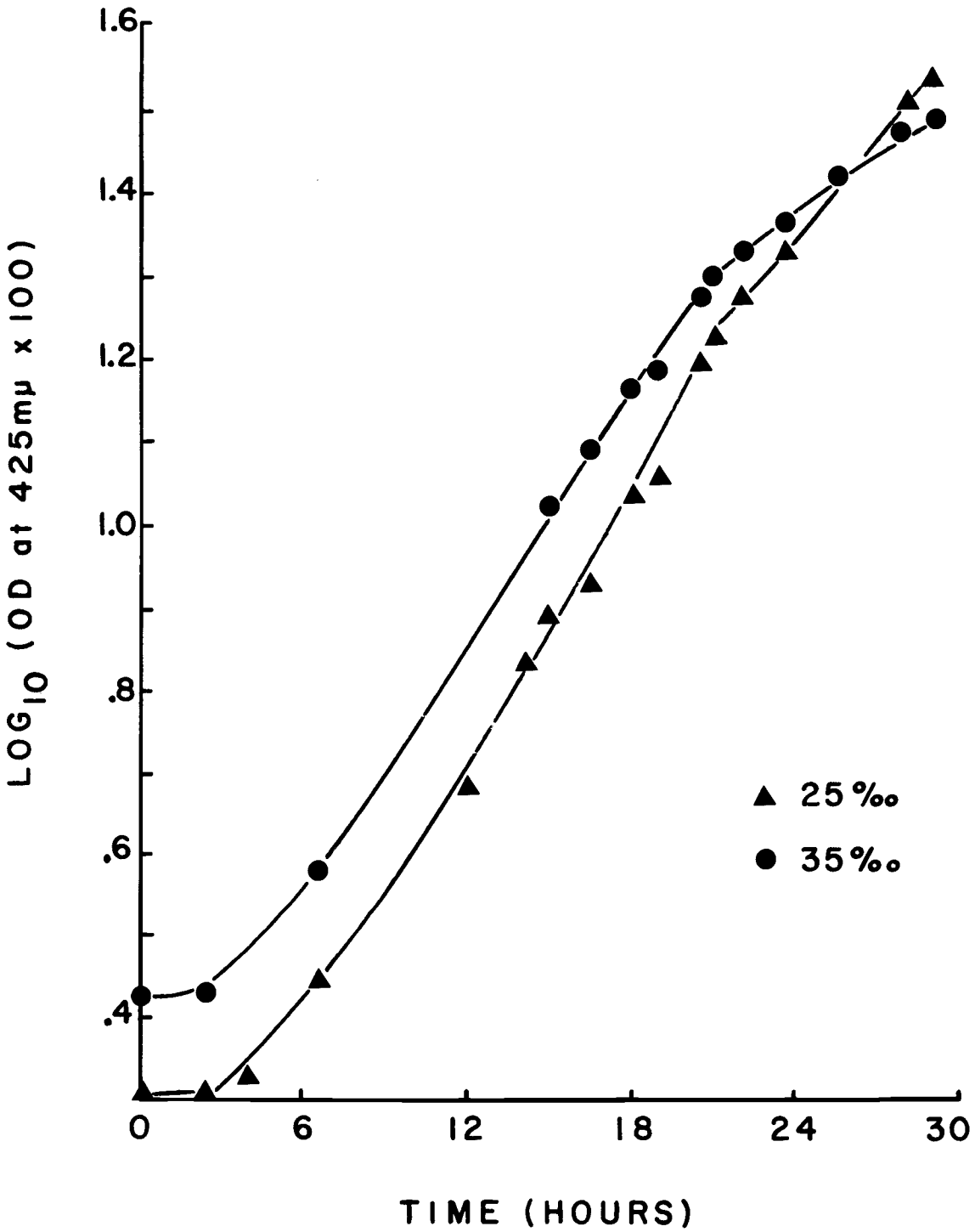

Figure 1. Growth curves of Vibrio marinus MP-1 in SSPU media at salinities of 25‰ and 35‰.

can vary considerably and thereby result in vastly different cell numbers corresponding to the same optical density of cell resuspensions (19). To obtain a constant number of resuspended cells for each salinity investigated, it was important to establish the relationship between optical density and total cell count of resuspensions made from cells grown at different salinities. OD vs cell numbers studies showed that counts of cell resuspensions in SSPU media at salinities between 25‰ and 40‰ resulted in correspondingly identical OD's of cell resuspensions. When the OD at 425 mμ varied from 0.125 to 0.175, the corresponding cell counts at all salinities varied from 1.7×10^7 cells/ml to 2.8×10^7 cells/ml, respectively. Cells grown at the lower salinities of 25‰ and 30‰ were short, semi-transparent rods, whereas cells grown at salinities of 35‰ and 40‰ were long, compact rods. The tendency for cells to become more spherical and less dense with decreasing salinity of the growth medium has been reported for halophilic bacteria (1, 73, 78). Several investigators suggest that in addition to effecting high osmotic pressure, Na^+ functions to maintain the integrity of the cell membrane by binding to neighboring negatively charged groups thus preventing their mutual electrostatic repulsion (40, 73). Divalent cations prevent dissociation by bridging neighboring negative charges, thus forming stronger bonds to protect the cell during osmotic pressure changes. The morphology change effected by decreasing the salinity

resembles the change resulting from incubating V. marinus MP-1 above the maximum temperature for growth. Likewise, Abram (1) observed that the lower the salt concentration in the medium, the shorter the time required for conversion of the rods of his red halophile to spheres by heating.

Comparative studies of protein synthesis in cells suspended in SSPU medium at 15°C, 20°C, and 25°C verified the results of previous protein synthesis determinations in Vibrio marinus (54). Incorporation of proline into protein during incubation of V. marinus MP-1 in SSPU (25‰) medium continued for 90 minutes at 15°C and 20°C but was negligible at 25°C (Fig. 2). Similar results were obtained from studies at salinities of 30‰, 35‰, and 40‰. At all four salinities, the rate of protein synthesis at 20°C was approximately equal to or greater than the rate at 15°C. These studies established that the critical temperature range of the thermal lesion in protein synthesis is between 20°C and 25°C for salinities of 25‰ to 40‰. Since cell growth at 15°C was extremely poor in SSPU (20‰) medium, comparative protein synthesis studies were not determined at this salinity.

Subsequent studies of protein synthesis at one degree temperature intervals in the 5°C critical range are reported for salinities of 25‰, 30‰, 35‰, and 40‰ (Figs. 3 - 6). Also included are the results of concurrent precursor uptake studies run on the protein

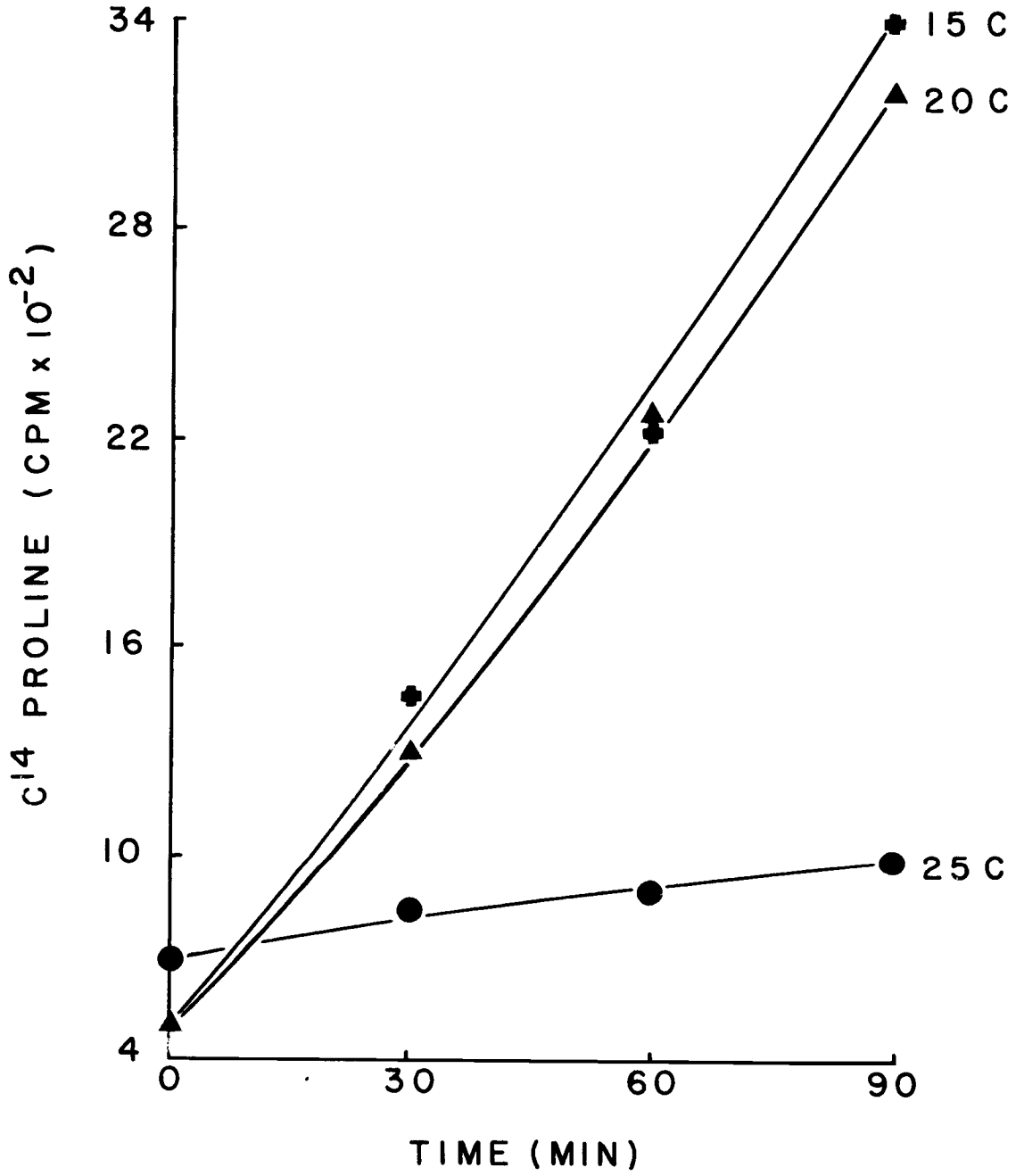

Figure 2. Incorporation of ^{14}C-proline into protein during incubation of Vibrio marinus MP-1 in SSPU (25‰) medium.

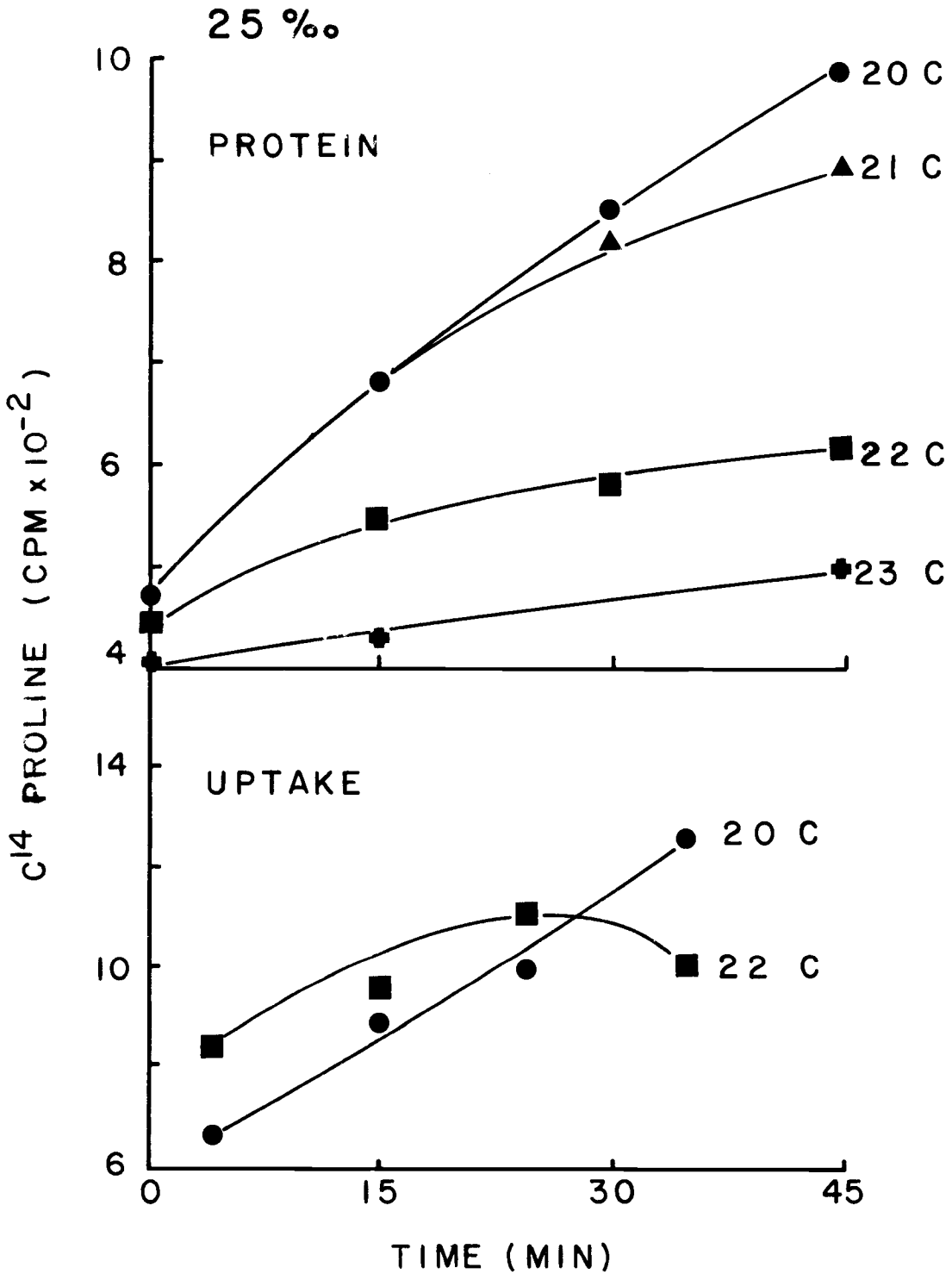

Figure 3. Incorporation into protein (top) and whole cell uptake (bottom) of ^{14}C-proline by Vibrio marinus MP-1 incubated in SSPU (25‰) medium.

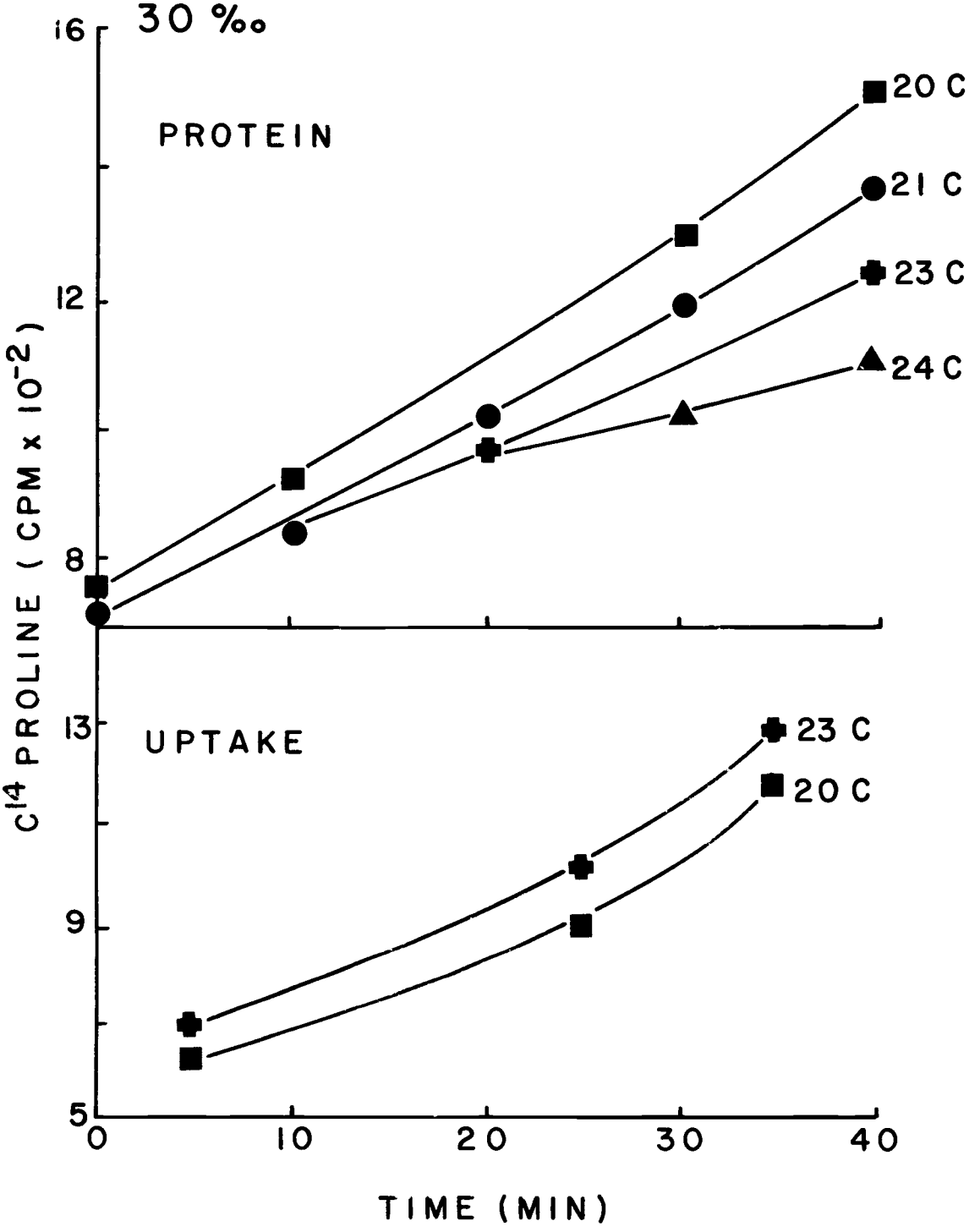

Figure 4. Incorporation into protein (top) and whole cell uptake (bottom) of ^{14}C-proline by Vibrio marinus MP-1 incubated in SSPU (30‰) medium.

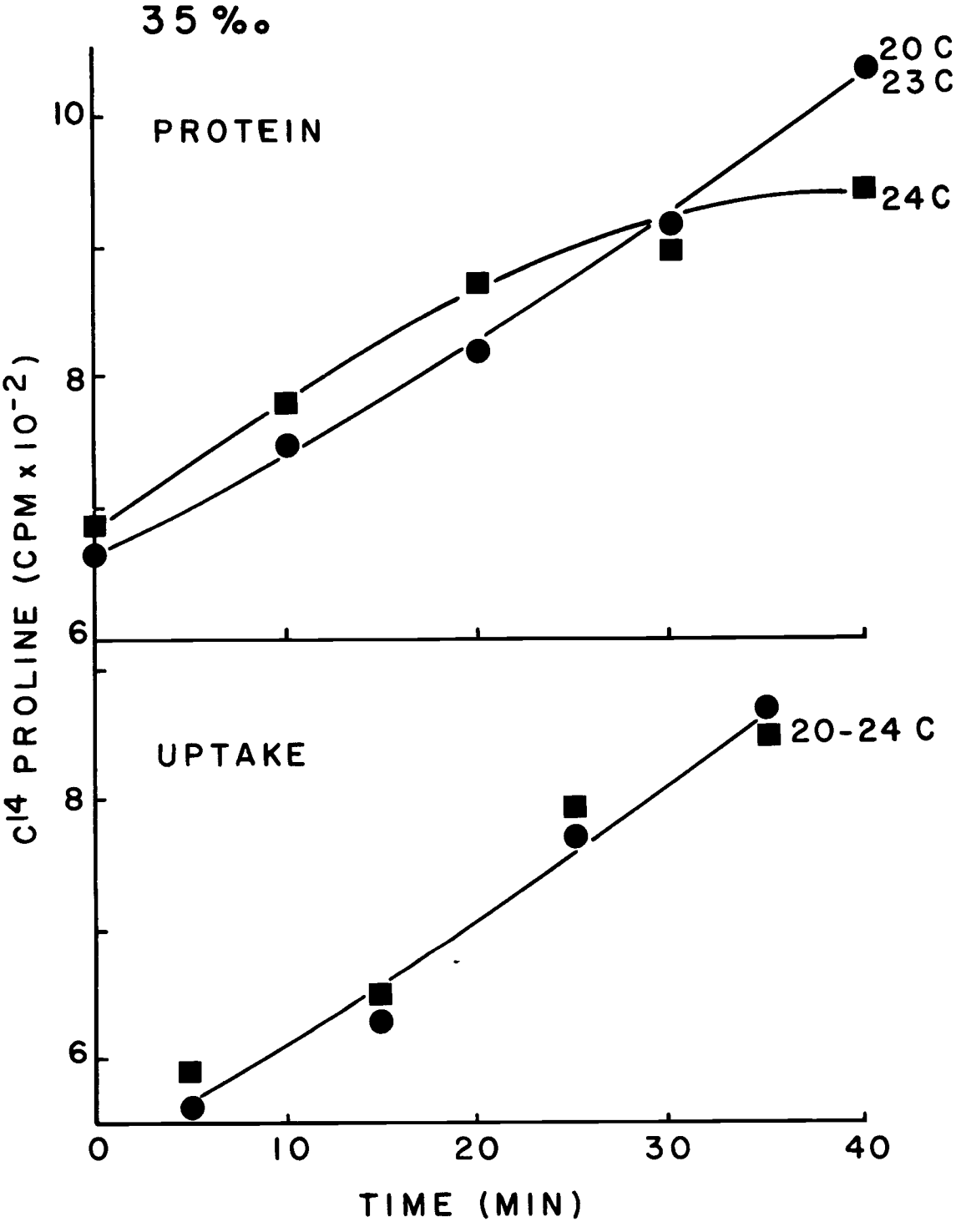

Figure 5. Incorporation into protein (top) and whole cell uptake (bottom) of ^{14}C-proline by Vibrio marinus MP-1 incubated in SSPU (35‰).

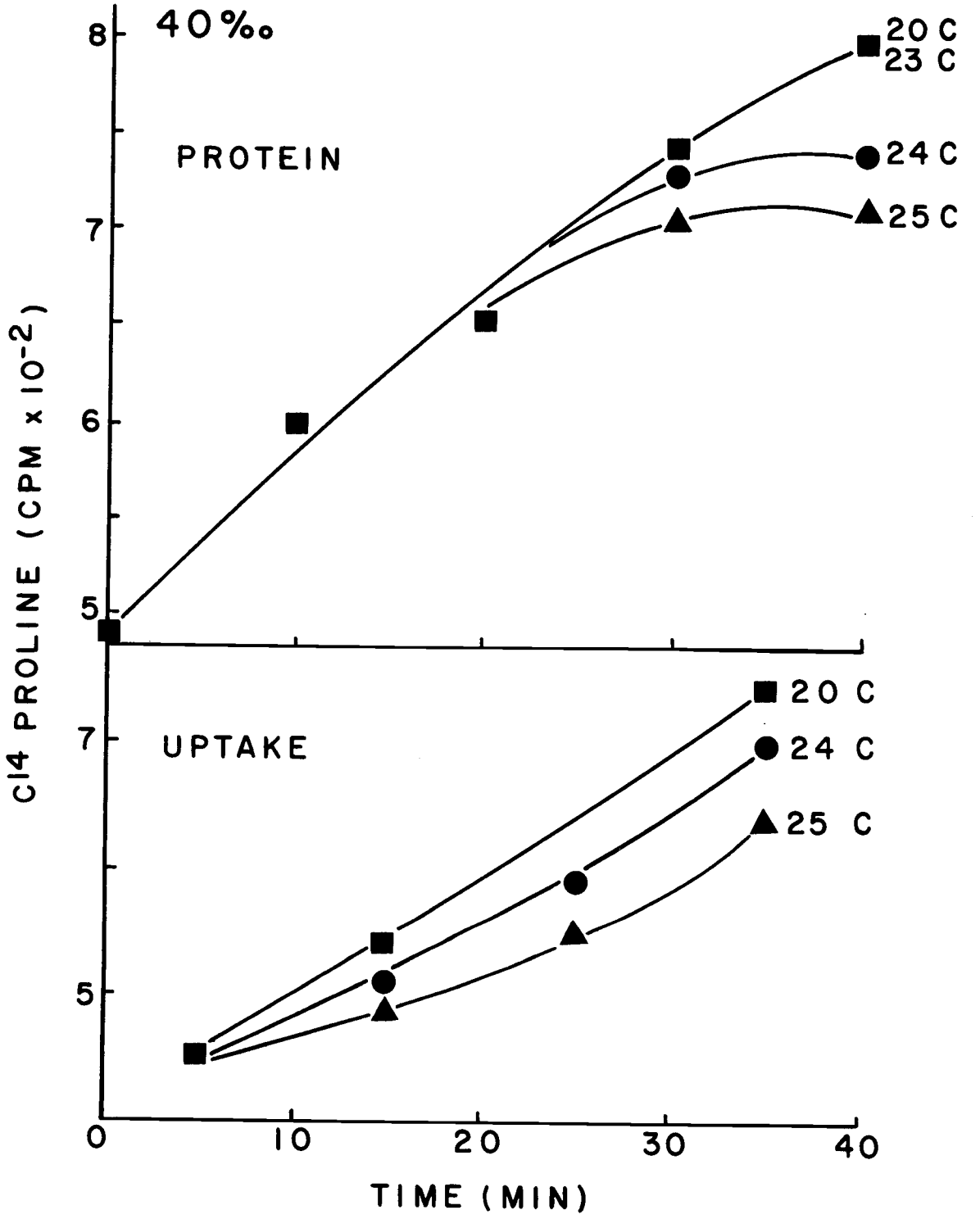

Figure 6. Incorporation into protein (top) and whole cell uptake (bottom) of ^{14}C-proline by Vibrio marinus MP-1 incubated in SSPU (40‰) medium.

synthesizing system. At salinities of 25‰ through 35‰, the uptake of precursor into whole cells continued after protein synthesis had decreased. At 40‰, the decrease in uptake of precursor at 24°C and 25°C is significant as compared to uptake at 20°C and preceeds by 25 minutes the decrease in incorporation of label into protein. A considerable decrease in protein synthesis occurs at 22°C in cells suspended in SSPU (25‰) medium (Fig. 3). In SSPU (30‰) medium (Fig. 4), the thermal inhibition of protein synthesis occurs in a gradual gradient of decreased rates between 20°C and 24°C, with protein synthesis still apparent after 40 minutes at 24°C. In SSPU 35‰ (Fig. 5), protein synthesis does not decrease until after 20 minutes incubation at 24°C. The uptake and protein synthesis studies (Figs. 3 - 6) may indicate that at different salinities of growth medium different mechanisms are involved in the inhibition of precursor incorporation into protein at 25°C. Since the decrease in uptake preceeds the decrease in incorporation of precursor into protein at 40‰ (Fig. 6), the apparent decrease in protein synthesis at 24°C may result from inhibition of cellular transport mechanisms. A salinity of 40‰ appears to prevent the uptake of extracellular amino acids at temperatures which did not prevent uptake at the lower salinity of 35‰ (Fig. 5). The lesion at 24°C in SSPU (40‰) medium may not directly involve the protein synthesizing system. At salinities of 25‰ to 35‰, precursor uptake continues after thermal inhi-

bition of protein synthesis thus suggesting a lesion in the cellular protein synthesizing system. The thermal lesion in protein synthesis occurs at lower temperatures in lower salinities of growth medium. Thus a temperature-salinity relationship is involved in the thermal inhibition of protein synthesis.

The site of the thermal lesion in cellular protein synthesis may be either in transcription of m-RNA, involving cellular control mechanisms, or in translation to protein involving heat sensitive synthetase enzymes, ribosomes or transferase enzymes. To determine which of these cellular components is initially inhibited by heat, the synthesis of RNA and protein were studied concurrently at 15°C and 22°C in SSPU media at salinities from 15‰ through 40‰ (Figs. 7 - 12). At salinities of 15‰ through 35‰, total RNA synthesis was greater at 22°C than at 15°C. Continued incorporation of uracil into RNA at 15°C and 22°C may indicate that no lesion occurs in uptake of this RNA precursor into whole cells. In SSPU (40‰) medium (Fig. 12), incorporation of uracil into RNA decreased after 10 minutes incubation at 22°C compared to RNA synthesis in the control at 15°C. This decrease may reflect either an inhibition of RNA synthesis at 22°C or an inhibition of uracil uptake similar to that observed for proline at this temperature and salinity. At 22°C, the decreased rate of protein synthesis in SSPU media at 25‰ (Fig. 9) and 30‰ (Fig. 10) and the continuation of protein synthesis at 35‰

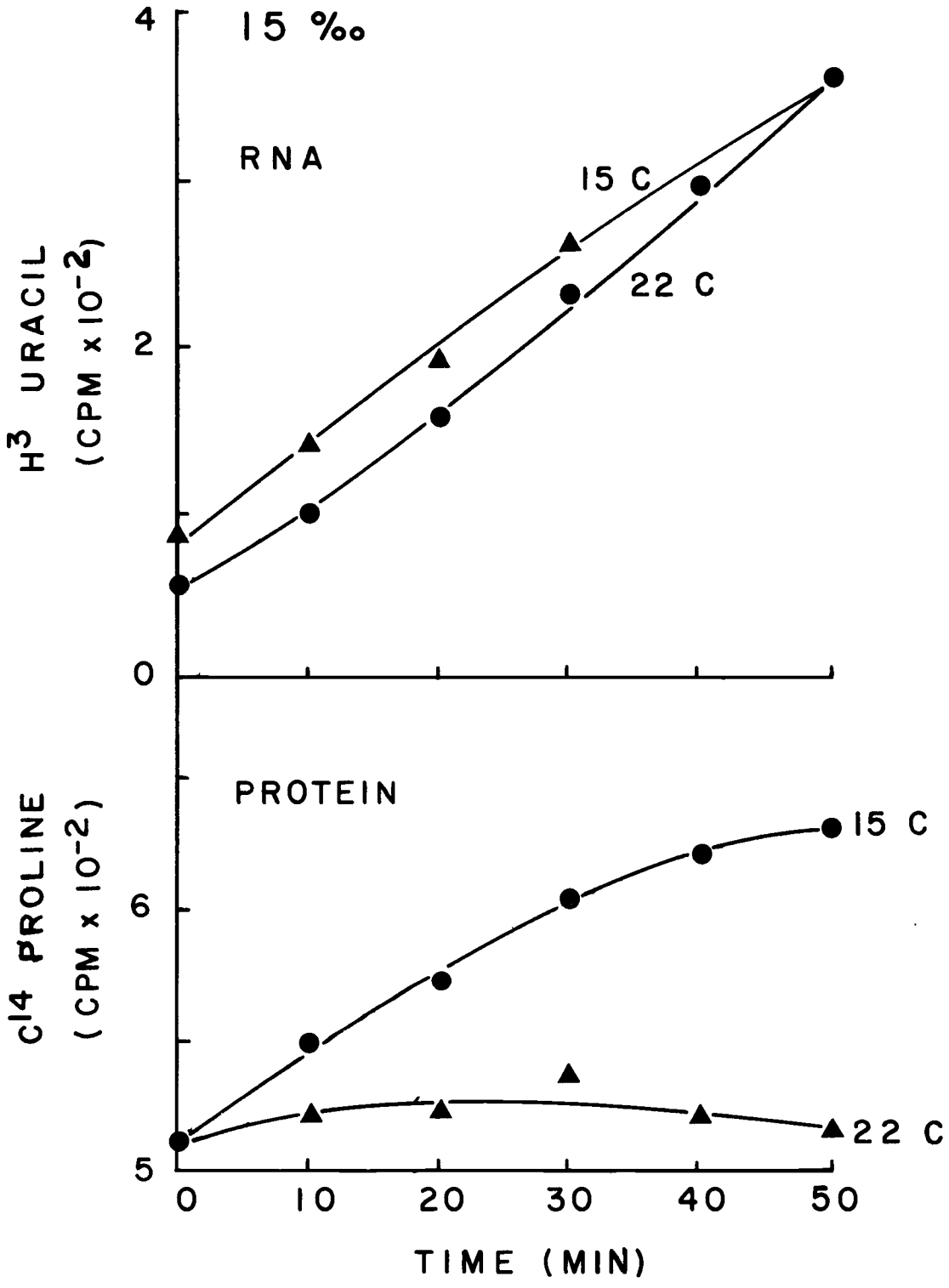

Figure 7. Incorporation of ^{3}H-uracil into RNA (top) and ^{14}C-proline into protein (bottom) during incubation of Vibrio marinus MP-1 in SSPU (15‰) medium.

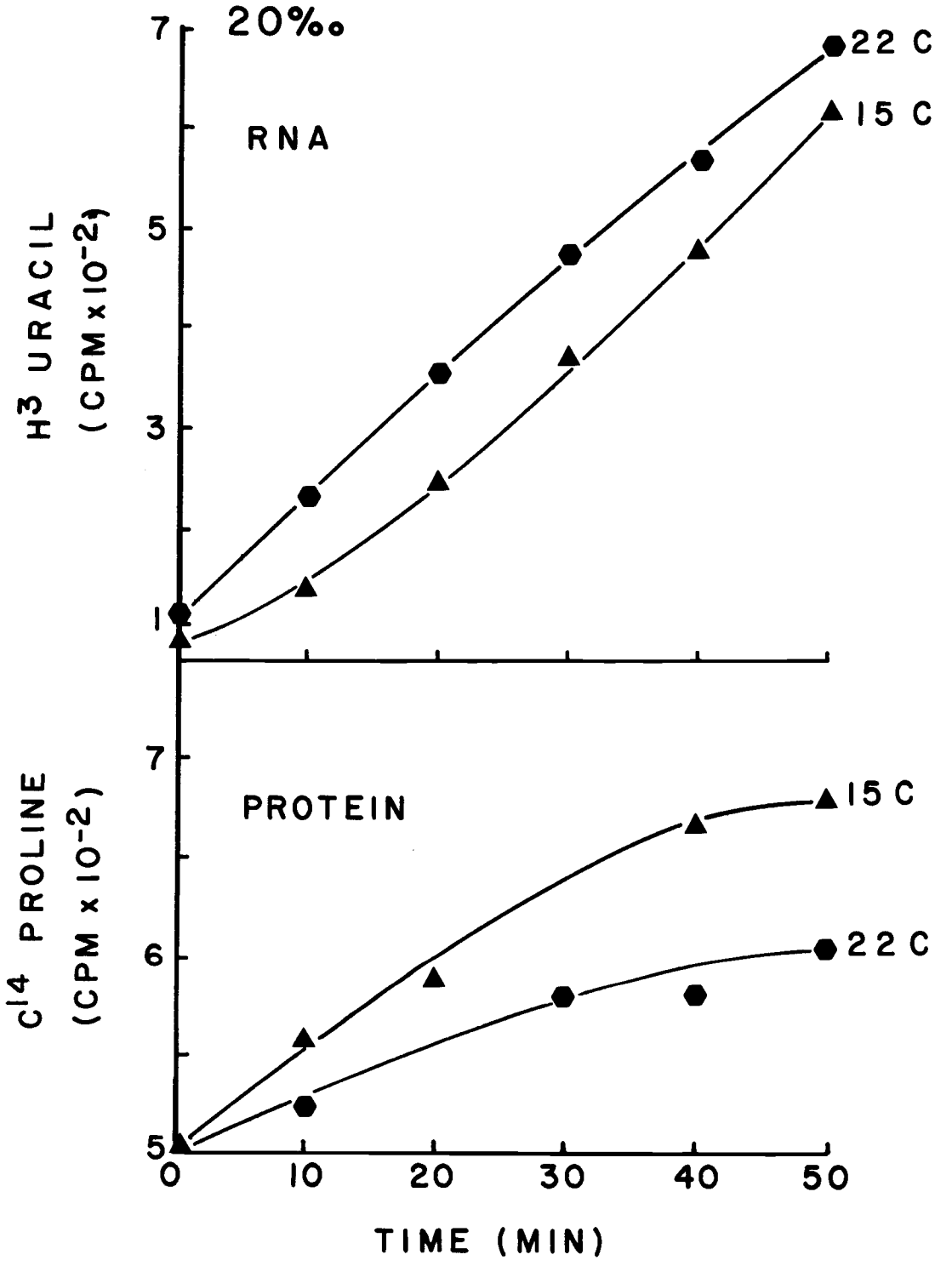

Figure 8. Incorporation of ^{3}H-uracil into RNA (top) and ^{14}C-proline into protein (bottom) during incubation of Vibrio marinus MP-1 in SSPU (20‰) medium.

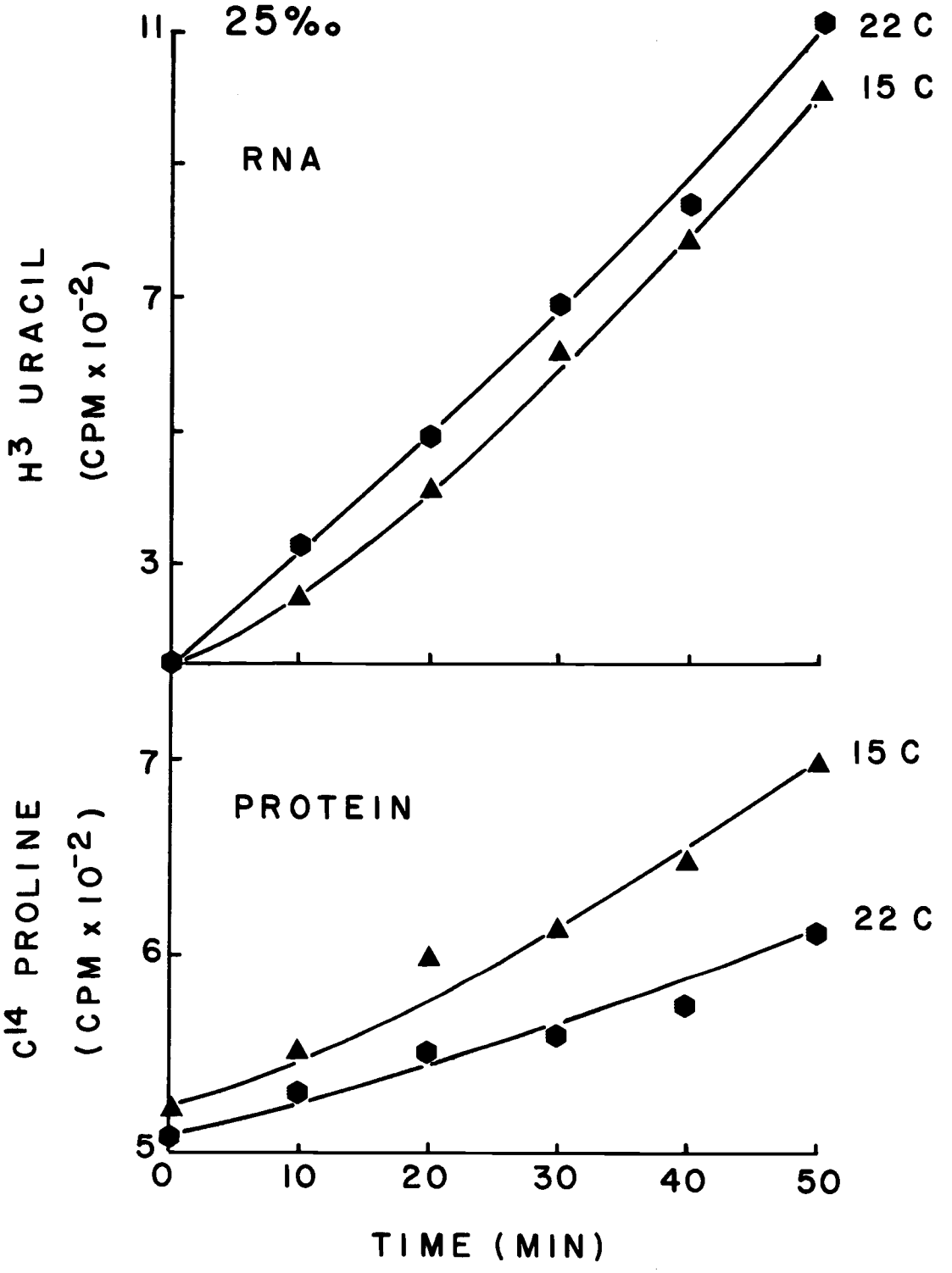

Figure 9. Incorporation of ^{3}H-uracil into RNA (top) and ^{14}C-proline into protein (bottom) during incubation of Vibrio marinus MP-1 in SSPU (25‰) medium.

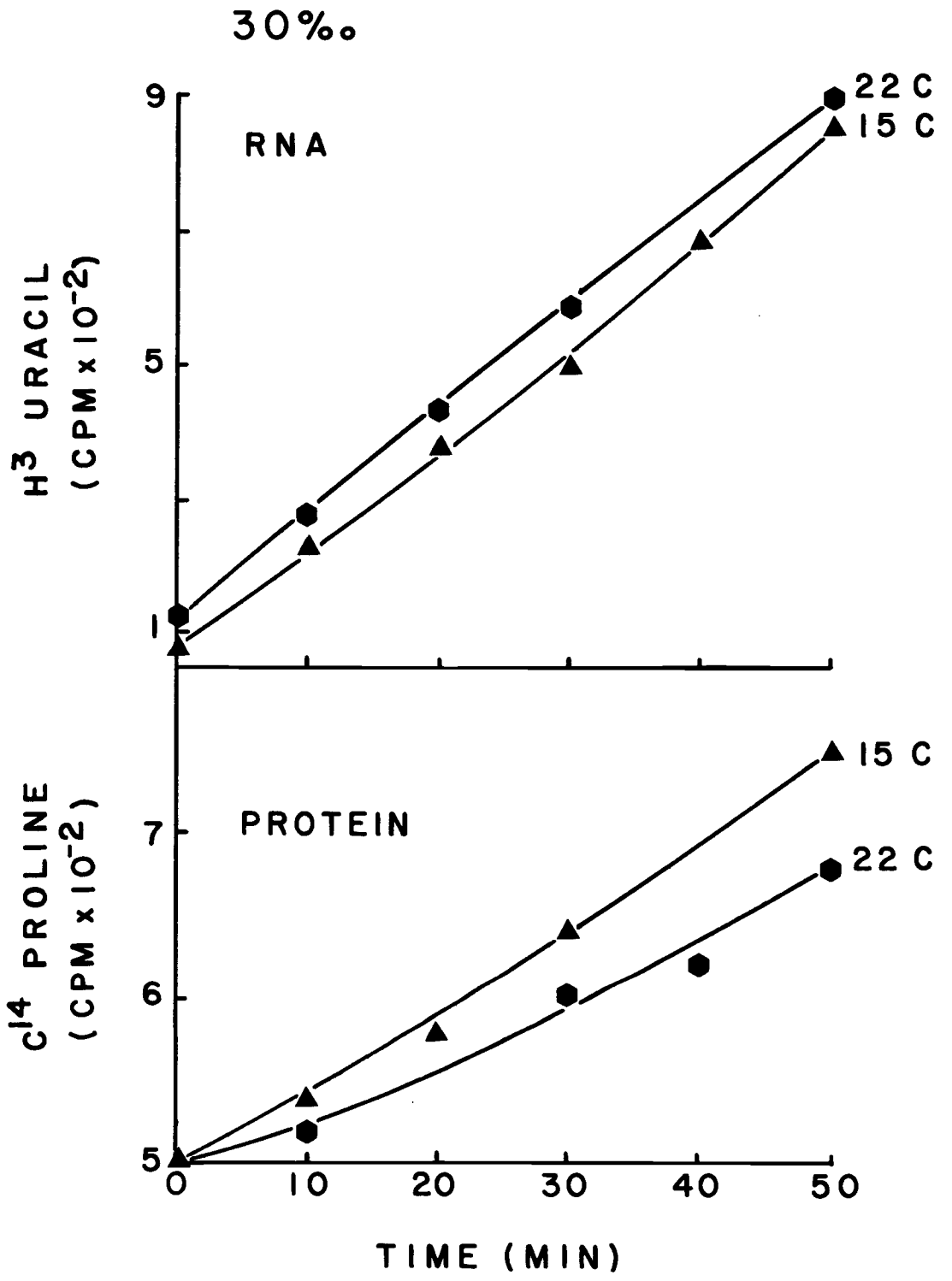

Figure 10. Incorporation of ^{3}H-uracil into RNA (top) and ^{14}C-proline into protein (bottom) during incubation of Vibrio marinus MP-1 in SSPU (30‰) medium.

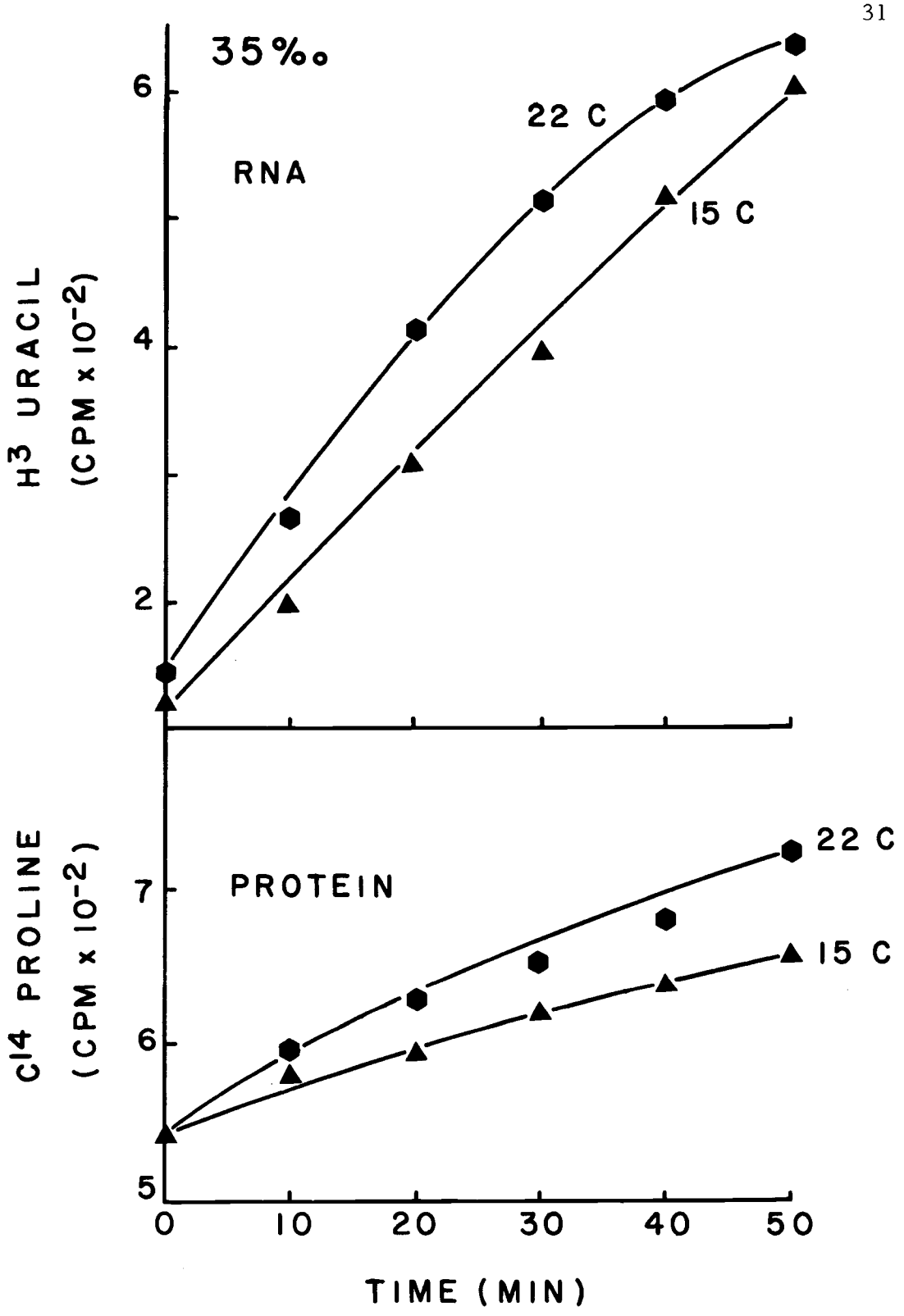

Figure 11. Incorporation of ^{3}H-uracil into RNA (top) and ^{14}C-proline into protein (bottom) during incubation of Vibrio marinus MP-1 in SSPU (35‰) medium.

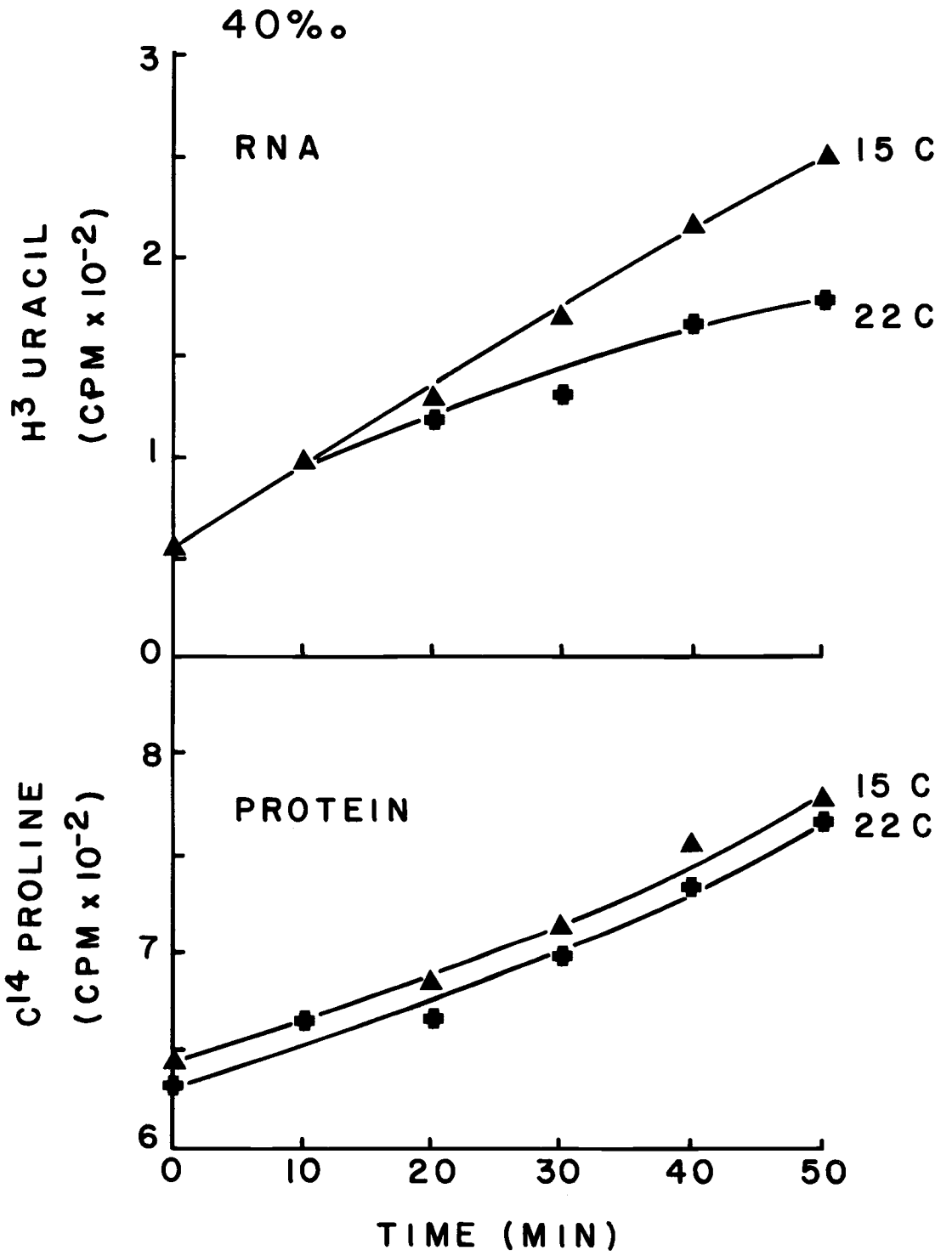

Figure 12. Incorporation of ^{3}H-uracil into RNA (top) and ^{14}C-proline into protein (bottom) during incubation of Vibrio marinus MP-1 in SSPU (40‰) medium.

and 40‰ duplicate the results of the critical temperature studies. In non-growth supporting SSPU (15‰) medium (Fig. 7), protein synthesis is negligible at 22°C but continues for 30 minutes at 15°C and then significantly decreases in rate. Similar results are seen in SSPU 20‰ (Fig. 8), but slight protein synthesis occurs for 30 minutes at 22°C. The decrease in the rate of protein synthesis at 15°C after 30 minutes incubation in SSPU media at 15‰ (Fig. 7) and 20‰ (Fig. 8) may either result in, or result from, the inability of cells to grow at these low salinities. Thus, at growth medium salinities of 15‰ to 30‰, although total RNA synthesis continues at both 15°C and 22°C, cellular protein synthesis is inhibited by either temperature or salinity effects. These results may explain the low maximum temperature for growth of 10.5°C demonstrated by Stanley and Morita (74) in V. marinus MP-1 cells in medium at a salinity of 10‰.

Although the uptake of l-proline is not initially inhibited at temperatures above 20°C in media at salinities of 25‰ to 35‰, it is possible that temperatures above the maximum for growth prevent the uptake of other required substrates. Several investigators demonstrated that some, but not all, cellular transport mechanisms in marine bacteria are Na^+ dependent (2, 9, 45). Furthermore, different concentrations of Na^+ are required for the activation of different cellular permeases. Although l-proline may enter cells and be available for protein synthesis at temperatures above the

maximum for growth, transport of the carbon source, succinate, may be thermally inhibited. After the utilization of intracellular reserves of succinate, the cells would stop synthesizing all cellular constituents, including amino acids, and a decline in protein synthesis would result. Although the data do not directly prove or disprove this possibility, it is likely that if the inhibition of succinate uptake prevents its utilization for further amino acid synthesis and subsequent incorporation into protein, a similar breakdown in the synthesis of nucleotides and their subsequent incorporation into RNA would occur. The continuation of RNA synthesis after protein synthesis has stopped indicates that a breakdown in nucleotide synthesis does not occur.

The correlation between the time of inhibition of protein synthesis and the onset of cell death was determined in SSPU at salinities of 25‰ (Fig. 13) and 35‰ (Fig. 14). The onset of cell death and the inhibition of protein synthesis both occur after approximately 40 minutes incubation at 22°C in SSPU (25‰) medium (Fig. 13). At 25°C in the same medium, both cell death and the inhibition of protein synthesis begins immediately. In SSPU (35‰) medium, the viability loss is very low at 22°C until after 2 hours incubation at which time the cells undergo a rapid loss of viability (Fig. 14). Concurrent protein synthesis at 22°C continues for two hours at a lower rate than in the control at 15°C and then completely stops at

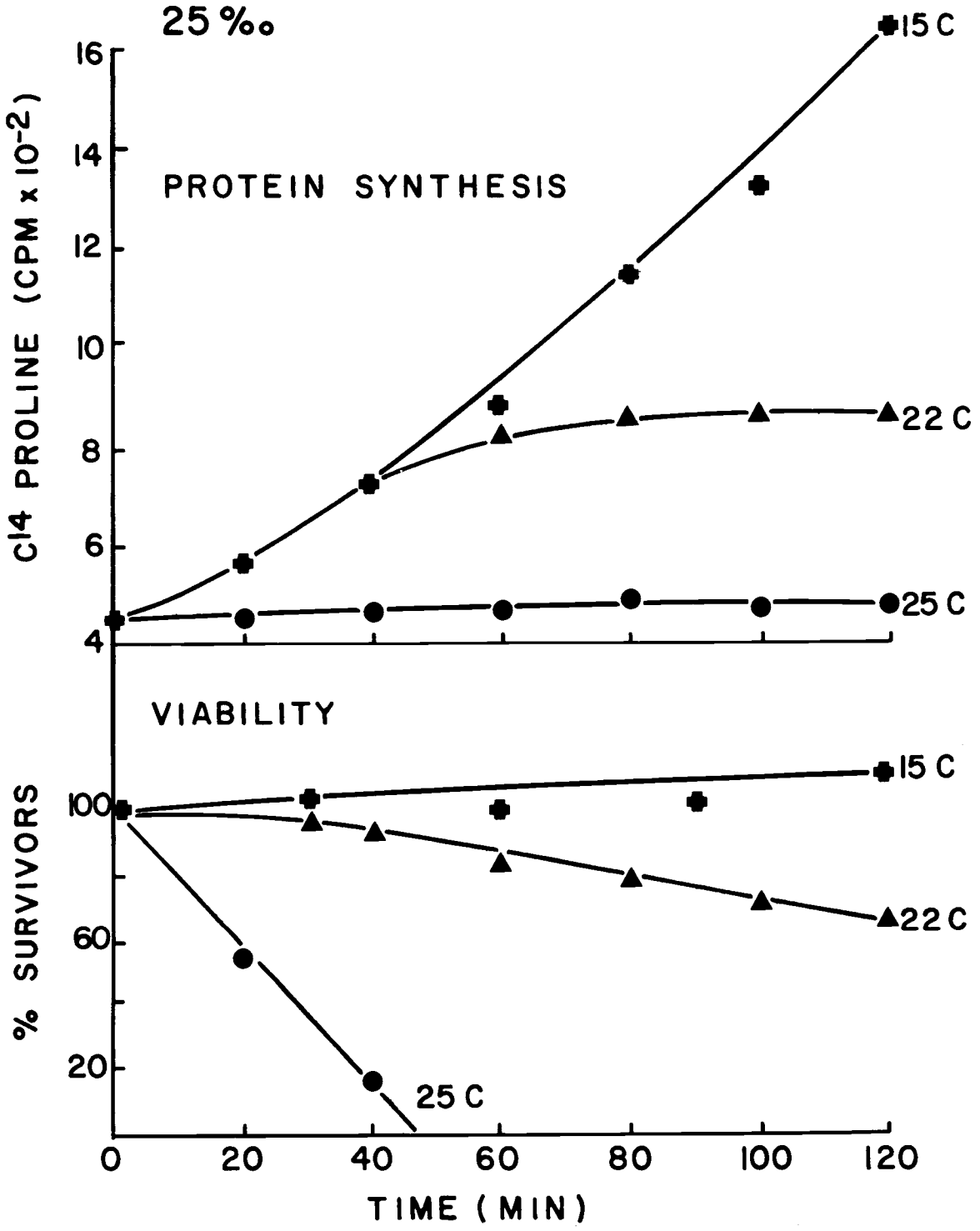

Figure 13. Protein synthesis (top) and cell viability (bottom) during incubation of Vibrio marinus MP-1 in SSPU (25‰) medium.

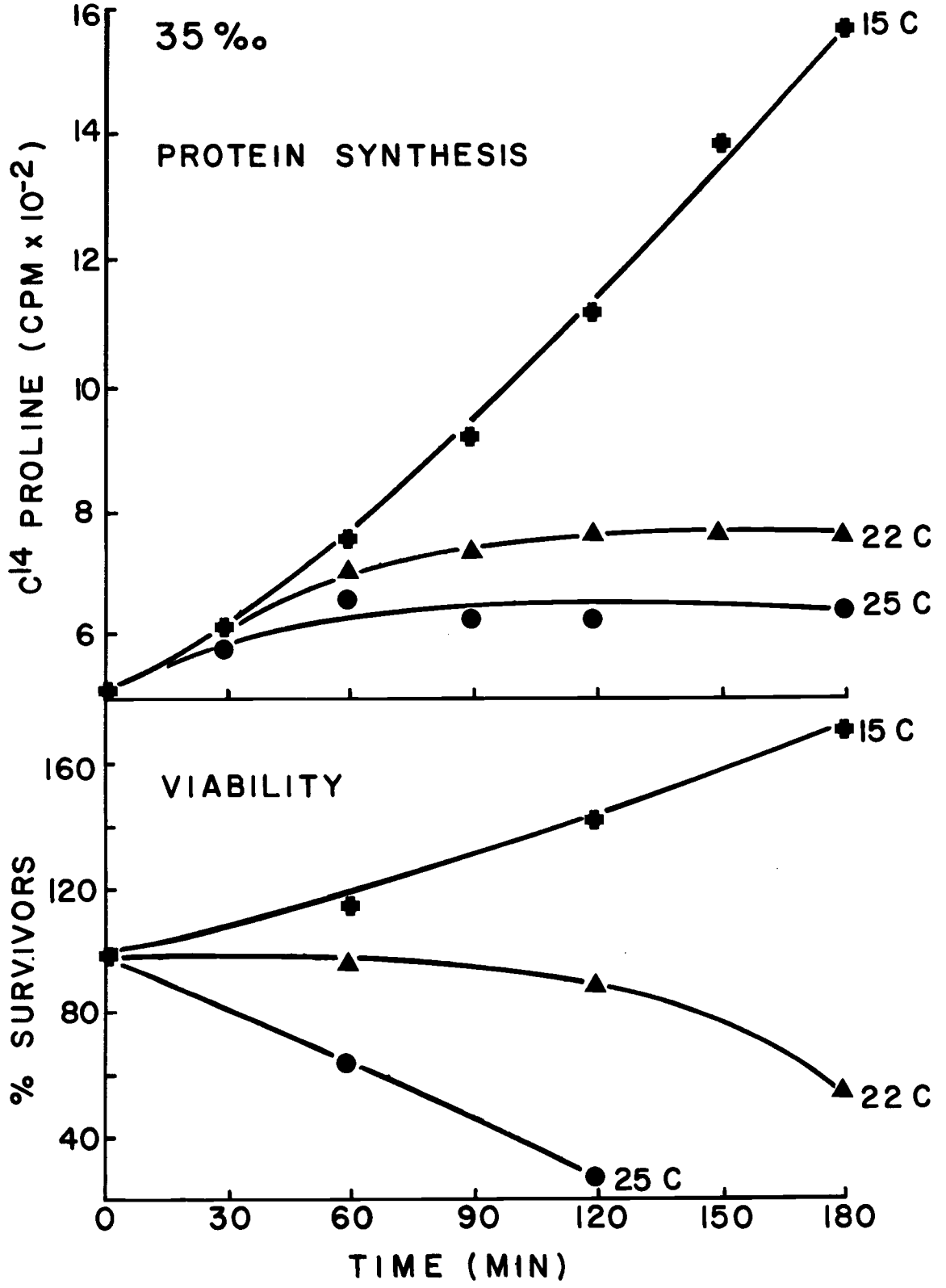

Figure 14. Protein synthesis (top) and cell viability (bottom) during incubation of Vibrio marinus MP-1 in SSPU (35‰) medium.

the same time the rapid loss of viability occurs. The loss of cell viability is immediate at 25°C in SSPU (35‰) medium and drops to 25 per cent of the original viability after two hours incubation. A low rate of protein synthesis occurs at 25°C for one hour and then stops completely. These data directly correlate the loss of cell viability with the thermal inhibition of protein synthesis at 25‰ and 35‰. Controls run at 15°C show continued cell viability and protein synthesis for the entire experimental period.

The data indicate that the thermal lesion in protein synthesis is at the translational level. Farrell and Rose (16) described enzymes as the most heat-labile cell constituents. In an obligate psychrophile, Malcolm (46, 48) demonstrated the temperature sensitivity of three aminoacyl-t-RNA synthetase enzymes (those for glutamic acid, histidine, and proline) and one t-RNA species (that cognate for glutamic acid). Heated psychrophile enzymes would not aminoacylate t-RNA molecules from any organism. However, heated psychrophile t-RNA was aminoacylated by heterologous mesophile or thermophile synthetase enzymes but not by its own unheated synthetase enzyme. Malcolm suggests that the conformation of t-RNA molecules changes with temperature, and the conformation of recognition sites for psychrophilic synthetase enzymes may differ from those for mesophilic or thermophilic enzymes. Sinclair and Grant (71) have reported a similar thermolabile receptor activity of the

t-RNA cognate for leucine from an obligately psychrophilic yeast exhibiting a thermal lesion in protein synthesis. Biochemical studies of Escherichia coli mutants with temperature sensitive phenylalanyl RNA synthetases demonstrated that the mutant enzymes consisted of two, non-catalytic sub-units, each one-half the size of the wild type synthetase (5). Böck suggests that the dissociation or weakening of sub-unit interactions causes the thermolability of this enzyme. In view of the previously discussed examples of the specific requirement for Na^+ (20, 43) and of the salt activation of enzymes from halophilic organisms (8, 9, 51, 66), it seems possible that thermally induced changes in structural and functional areas of synthetase enzymes or t-RNA molecules may be protected by the binding of Na^+ ions to these molecules. The reduction of intramolecular electrostatic repulsion by salt may prevent the weakening of thermally increased intramolecular electrostatic repulsive interactions and subsequent inactivating conformational changes.

Since all RNA synthesis studies in these experiments determined only total RNA synthesis, it is possible that the lesion may be at the transcriptional level by preventing the synthesis of m-RNA while allowing the synthesis of other RNA species to continue. This would result in a subsequent decrease in the synthesis of cellular protein. Since turnover of m-RNA occurs in protein synthesizing cells and the amount of m-RNA present in cells at any time may be

low compared to the amount of total cellular RNA, differences in the amount of m-RNA synthesized at 22°C and 15°C may not be detected in the experimental system.

A number of cellular control mechanisms may be implicated in the thermal damage to V. marinus. Several investigations have demonstrated temperature sensitive regulatory systems (18, 31). Gallant and Stapleton (18) showed that a system controlled at low temperatures by inorganic phosphate repression undergoes constitutive alkaline phosphatase synthesis at high temperatures due to a thermosensitive aporepressor synthesizing system. It is notable that in the comparative protein synthesis studies reported here for salinities of 25‰ to 40‰, often more total protein was synthesized at 20°C than at 15°C, the optimum temperature for growth of the organism. More total RNA was also synthesized at 22°C than at 15°C at salinities of 15‰ through 35‰. These results are similar to those reported by Harder and Veldkamp (27) in their continuous culture study of an obligate psychrophile. At temperatures above the optimum for growth, more total RNA and protein were synthesized to compensate for thermally impaired protein synthesis. The optimum temperature for respiratory enzyme activity was above the maximum growth temperature of the organism. In whole cell studies of V. marinus, the synthesis of more total protein at 20°C than at 15°C may result from the synthesis of more total RNA in response

to both the partial thermal inactivation of protein synthesizing systems and the inactivation of cellular enzymes.

The thermal stability of V. marinus ribosomes and ribosomal RNA up to an incubation temperature of 69° C (62) accompanies the thermostability of RNA forming systems above the maximum temperature for growth. However, both the protein synthesizing system and several cellular enzymes appear to be thermolabile at temperatures above the maximum for growth. Although the maximum activity of some psychrophilic enzymes may occur at temperatures far above the maximum temperature for enzyme production (60, 80, 81, 82), the continued growth and metabolism of these organisms in the environment may be thermally inhibited.

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