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MACROMOLECULAR SYNTHESIS IN *LEUCOSPORIDIUM STOKESII* DURING HEAT INJURY AND RECOVERY

by

John William Spencer

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY AND MEDICAL TECHNOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY
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GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by John William Spencer

entitled Macromolecular Synthesis in Leucosporidium stokesii During Heat Injury and Recovery

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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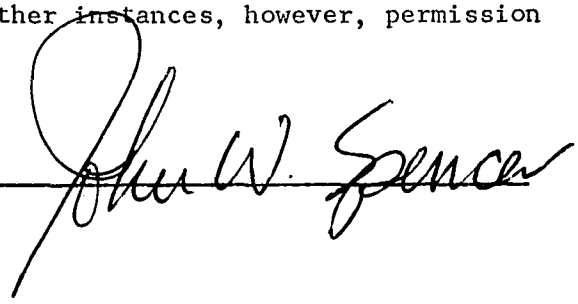
SIGNED: 

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ABSTRACT

Cells of three obligately psychrophilic yeasts, Leucosporidium frigidum, Leucosporidium stokesii, and Candida sp. P-25, were subjected to heat stress at temperatures from five to ten degrees above the maximum growth temperatures of the organisms. Measurements and comparisons of their responses to heat stress were made and correlated with previously published data on thermal injury and thermal death. Among the parameters measured were viability, respiratory activity, and leakage of ultraviolet absorbing material from the cell. Both L. frigidum and Candida sp. P-25 lost respiratory activity to a greater degree than L. stokesii under the same conditions of heat stress. Differences in response to heat treatment were also noted with respect to viability and OD_{260} leakage.

The response of L. stokesii to heat stress was more closely investigated. Cells were subjected to heat of 25°C and 30°C (five and ten degrees above the maximum growth temperature, respectively), and response was measured in terms of whole cell uptake of radioactive macromolecular precursors, incorporation of radioactive macromolecular precursors into major cell fractions, chemical determinations of RNA, DNA, and protein synthesis, ATP levels, and recovery of the cell membrane upon exposure to permissive temperature after heat stress.

Uptake to macromolecular precursors was profoundly affected by heat. Uptake of uracil and thymine was initially stimulated followed by release of radioactivity back into the medium. Leucine uptake

rapidly ceased upon exposure to 30°C heat and was markedly reduced by exposure to 25°C heat. Chemical determinations of macromolecular synthesis showed that protein was being produced in the cell at 25°C throughout the stress period and for a short time when the stressing temperature was 30°C. The DNA production was very slightly stimulated for a short initial period at both stressing temperatures but remained constant over the remainder of the stressing period. Chemical determinations of RNA content showed a pattern of response identical to the viability curves obtained with whole cells. At 25°C a large initial stimulation of RNA production was noted for the first hour of treatment after which levels of RNA decreased as the viability of the cells decreased. At 30°C RNA content of the cells decreased from the beginning of heat stress as did viability.

Both chemical and tracer data indicated that synthetic activity of the cell increased upon exposure to 25°C and 30°C. The ATP levels during heat stress correlated well with this hypothesis as they declined rapidly during the initial stages of heat stress and actually increased when chemical and tracer data indicated that synthesis had stopped.

Previous data indicated that protein synthesis was the heat-sensitive cellular process responsible for thermal death in L. stokesii. At 25°C protein synthesis was found to be unimpaired and only RNA content, of all the parameters measured, correlated with the loss of viability of L. stokesii during exposure to supermaximal growth temperatures.

INTRODUCTION

Psychrophilic organisms are widespread in nature. The majority of psychrophiles thus far studied are bacteria although psychrophilic yeasts, molds, and algae have been isolated. The most frequently isolated psychrophilic bacteria are species of the genera Pseudomonas, Flavobacterium, Alcaligines, Achromobacter, and Arthrobacter. To a lesser extent psychrophilic species of the genera Escherichia, Aerobacter, Aeromonas, Serratia, Proteus, Chromobacter, and Vibrio have been isolated. Psychrophilic clostridia, Bacillus, Lactobacillus, Cornynebacterium, and Micrococcus have also been isolated. The most frequently isolated psychrophilic yeasts belong to the genus Candida, although psychrophilic Cryptococcus, Rhodotorula, and Torulopsis species have been studied. Molds which exhibit the ability to grow at 0°C include species of Pullularia, Botrytis, and Geotrichum. Several genera of algae have also been shown to be psychrophilic (Farrell and Rose 1967).

The definition of the term psychrophile has evolved as more and more organisms have been isolated and studied. Microorganisms are classified in part according to their cardinal growth temperatures; i.e., their minimum, maximum, and optimum growth temperatures, as mesophiles, thermophiles, and psychrophiles. In general, mesophiles grow over the range of 15°C to 45°C with the optimum falling between 20°C and 40°C. Thermophiles grow over the range of 35°C to 100°C with the

optimum for growth between 55°C and 60°C. Psychrophiles grow over the range of 0°C to 30°C with the optimum growth temperature between 20° to 25°C. Since many psychrophiles grow optimally at temperatures in the mesophilic range, Ingraham and Stokes (1959) suggested that psychrophiles be defined on the basis of their ability to grow at 0°C. Their definition was based on the production of visible growth in one to two weeks at 0°C. Stokes (1963) also proposed that the optimum growth temperature be used to separate obligate psychrophiles from facultative psychrophiles. Thus obligate psychrophiles grow optimally below 20°C and facultative psychrophiles grow optimally above 20°C (Stokes 1963). For this discussion the definition of Stokes (1963) will be used.

The importance of psychrophilic organisms has been more generally recognized in the last fifteen years as the use of low temperature storage for preservation of foodstuffs has become more common. Also, the recent interest in the physiology and ecology of microorganisms inhabiting naturally cold environments has contributed to an increased interest in psychrophiles. Over 95% of the world's oceans by volume are below 5°C (Morita 1967). Moreover, more than 15% of the world's land mass has an average temperature below 2°C (Brock 1967).

The majority of studies on the physiology of psychrophiles have been undertaken because of their ability to grow at temperatures below the range of growth for most organisms as well as their inability to grow at even moderately elevated temperatures. The ability to grow at 0°C and indeed even lower if the medium can be kept in the liquid state is well documented (Straka and Stokes 1960, Sinclair and Stokes 1965,

Ahern and Roth 1966, diMenna 1966, Larkin and Stokes 1968). It has been suggested that factors involved in the determination of minimum growth temperatures might include the degree of saturation of lipids in the cell (Marr and Ingraham 1962). Kates and Baxter (1962) showed an increase of nearly 50% in the number of unsaturated bonds in lipids of mesophilic and psychrophilic yeasts grown at 10°C as opposed to 25°C. Wells, Hartsell, and Stadelman (1963) however found no changes in lipid composition of psychrophilic bacteria when growth temperature was shifted down during growth. Meyer and Bloch (1963) showed that cell-free extracts of Candida utilis which convert oleic acid to linoleic acid are much more active in this respect when the organism is grown at a lower temperature. It is known that an increase in the number of unsaturated bonds in a lipid leads to a decrease in the melting point of the lipid and thus could have a profound effect on the physiology of the cell. It is suggested that this increase in unsaturated lipids may act to maintain the lipid in a liquid state at low temperatures and thus allow membrane function at the lower temperature. This theory is called the "lipid solidification theory" and was developed by Gaughren (1947), Allen (1953), Farrell and Rose (1967), and Byrne and Chapman (1964). Since most, and sometimes all, of the lipid in a cell is associated with the cell membrane, these observations point to the cell membrane as a primary reason for the ability of psychrophiles to grow at lower temperatures than other organisms.

Concomitant with the "lipid solidification theory" is another hypothesis which involves the cell membrane. The latter hypothesis

involves the ability of psychrophiles to transport solutes into the cell at low temperatures and was proposed by Ingraham and Bailey (1959) for a pseudomonad. Sizable differences between the ability of whole cells of mesophiles and psychrophiles to oxidize glucose at low temperatures were noted. Mesophilic organisms were much less efficient at glucose oxidation at low temperatures than the psychrophile. No differences were found in temperature coefficient between the two organisms when crude extracts were used. Baxter and Gibbons (1962) compared mesophilic and psychrophilic yeasts and reported that the rate of sugar transport in the psychrophile was virtually independent of temperature from 0°C to 30°C. Sugar transport in the mesophile, however, showed a great dependence on temperature. Cirillo, Wilkins, and Anton (1963) observed that glucosamine uptake and glucose utilization were more affected at low temperatures in the mesophile, Saccharmyces cereviseae than in the psychrophilic Candida sp. It was observed that the temperature coefficient for glucose utilization for the psychrophile is constant from 0°C to 30°C while it varies from 50,000 between 0°C and 10°C to 17,000 between 10°C and 30°C for the mesophile. Cells of both organisms were ruptured and the activity of hexokinase was compared. When the cells were ruptured no difference was observed between the temperature characteristics of the enzyme from the two organisms. Rose (1968) suggests that these observations may be related in that the fatty acid side chains in the membrane lipids have an effector action for permases. Farrell and Rose (1968) have shown the solute transporting activity of membrane proteins can be influenced by the degree of

saturation of the fatty acid chains of membrane lipids. The above observations implicate permeability of the cell membrane in the unique ability of psychrophiles to exhibit a lower minimum growth temperature than their mesophilic counterparts.

A second theory to account for the low minimum growth temperature involves metabolic regulation of the cell. The regulatory mechanisms of psychrophiles are thought to be less sensitive to cold than those of mesophiles. Microbial metabolism operates under many control mechanisms including end product inhibition, catabolite repression, feedback inhibition, allosteric activation, and others. These mechanisms insure that the cell produces just sufficient intermediates and end products to satisfy its immediate metabolic demands (Rose 1963). It has been reported (Halpern 1961) that temperature has an effect on the control systems of E. coli in that synthesis of glutamate decarboxylase is inducible at 37°C but partly constituative at 30°C. In addition, Szer and Ochoa (1964) and Freidman and Weinstein (1964) have shown that in thermophilic organisms temperature influences the fidelity of translation of mRNA. Marr, Ingraham, and Squires (1964) have shown that the affinity of an allosteric protein for a low molecular weight compound can be greatly affected by temperature. Quist and Stokes (1969) observed in a psychrophilic bacterium that the induction of formic hydrogenlyase was possible over the range of 0°C to 20°C and that the same enzyme in the mesophile used for comparison was noted over the range of 15°C to 45°C. These observations imply that the ability of the psychrophiles to grow at low temperatures is complex.

Several factors which determine the low maximum growth temperature have also been studied. Many of the constituents of the psychrophilic cell have been shown to exhibit sensitivity to heat. The heat-sensitive systems include enzymes (Upadhyay and Stokes 1963a,b; Purohit and Stokes 1967; Stokes 1967; Grant, Sinclair, and Nash 1968; Nash, Grant, and Sinclair 1969; Malcolm 1969), protein synthesis (Harder and Veldkamp 1967; Malcolm 1968; Nash, Grant, and Sinclair 1969), RNA synthesis (Malcolm 1968; Harder and Veldkamp 1968; Clark, Witter, and Ordal 1968; Allwood and Russell 1968), DNA synthesis (Harder and Veldkamp 1967), and membrane failure (Robinson and Morita 1966; Allwood and Russell 1968; Harder and Veldkamp 1968; Malcolm 1968; Hagen, Kushner, and Gibbons 1964; Nash and Sinclair 1968; Haight and Morita 1966; Kenis and Morita 1968).

One widely accepted explanation of the inability of psychrophiles to grow at elevated temperatures is that of denaturation of heat-sensitive enzymes or heat-induced cessation of enzyme synthesis. Hagen and Rose (1961,1962) transferred a growing culture of a psychrophilic cryptococcus from 16°C to 30°C. Growth ceased at 30°C after intracellular pools of amino acids and keto acids were depleted. The addition of several TCA cycle intermediates stimulated growth. These observations suggested that the cell was unable to synthesize certain essential intermediates at the restrictive growth temperature. These data further suggested that the enzymes for their production were inactivated. More recently, Evison and Rose (1965) showed that the activities of some of the TCA cycle enzymes decreased when psychrophilic

species of Arthrobacter and Candida were subjected to temperatures 3°C to 5°C above their maximum growth temperature. Baxter and Gibbons (1962) reported that the activity of alcohol dehydrogenase, respiration of intact cells, and the entry of glucosamine into the cell were all heat-sensitive in a psychrophilic Candida species. Upadhyay and Stokes (1963a) demonstrated a temperature-sensitive formic hydrogenlyase in the psychrophile strain 82. The enzyme was shown to be most active at 30°C and completely inactive at 45°C. The enzyme isolated from the mesophile E. coli had optimum activity at 45°C and was inactivated at 70°C. In addition, the lyase synthetic system was shown to be inactivated at temperatures above 20°C. The same authors (1963b) reported that hydrogenase and its synthesis were more heat-sensitive than those of E. coli. Purohit and Stokes (1967) reported that the oxidative and fermentative activity of a psychrophile were completely destroyed while activity of the mesophile E. coli was unaffected. Similar results were obtained with cell-free extracts and thus the complicating effect of cell permeability was removed. Indirect evidence for enzyme sensitivity at restrictive growth temperatures was provided by Burton and Morita (1963) and Morita and Burton (1963). These investigators showed that malic dehydrogenase activity of a marine psychrophilic, bacterium Vibrio marinus, was inactivated at or near the maxima growth temperature. Ogata et al. (1969) showed that l-alanine dehydrogenase activity occurred at 5°C in a psychrophilic brevibacterium but not at 28°C. Glucose fermentation by the obligate psychrophile Leucosporidium stokesii (formerly Candida gelida) was shown to be sensitive to 35°C

heat (Sinclair and Stokes 1965). This sensitivity was shown to be due to the possession of a heat-sensitive pyruvate decarboxylase (Grant, Sinclair, and Nash 1968). These examples amply demonstrate that cessation of growth at moderate temperatures of psychrophiles may be owing to the possession of heat-sensitive enzymes involved in part in energy transformation.

Since enzymes are composed of proteins, the investigation of protein synthesis as a determining factor of the maximum growth temperature of psychrophilic organisms has also been undertaken. Malcolm (1968) studying a psychrophilic micrococcus showed that rapid loss of viability occurred when exponential phase cells were transferred to a restrictive growth temperature, viz. 25°C. It was concluded that the loss of viability was not due to membrane damage, inhibition of respiration or of energy metabolism, or depletion of intracellular reserves. Suspensions of cells unable to form colonies showed an increased level of intracellular ATP and amino acids and also leakage of degraded RNA products into the medium. Uptake and incorporation studies employing radioactive macromolecular precursors indicated an inhibition of protein synthesis followed by inhibition of ribonucleic acid synthesis. Deoxyribonucleic acid synthesis was unaffected at temperatures above the maximum growth temperature. Malcolm (1969) further defined the primary lesion in Micrococcus cryophilus as a temperature sensitivity of two amino acid activating enzymes and their cognate species of t-RNA (glutamy-t-RNA synthetase and prolyl-t-RNA synthetase). These enzymes were shown not only to be temperature-sensitive but also able to

recognize their cognate t-RNAs only when the latter were in a low temperature configuration. This suggested that the t-RNA would not accept amino acids after heat treatment. If charging of the t-RNA had already taken place, no effect on the activity of the charged molecule was observed. Malcolm contended that the heat-induced lesion in protein synthesis was the primary lesion which resulted in death of the cells at moderately elevated temperatures. Similar observations by Nash, Grant, and Sinclair (1969) with the obligately psychrophilic yeast C. gelida (Leucosporidium stokesii) showed that in a cell-free system the amino acid incorporating system was completely inhibited after incubation at 35°C for 30 min. The enzymes implicated in the loss of protein synthetic ability were the amino-acyl-sRNA synthetases. Thirteen of these enzymes were examined and seven of them retained less than 50% of their activity after the heat treatment. None of the ten sRNA species which were tested showed any temperature sensitivity. Evidence was also presented which suggests that enzymes involved in the binding of polypeptide chains to the ribosomes are also heat labile. When the system was incubated at 25°C rather than 35°C only a slight reduction of amino acid incorporation was noted. Nash and Grant (1969) reported that the obligately psychrophilic yeast Candida gelida (L. stokesii) possessed ribosomes which were rendered completely nonfunctional after exposure to 40°C for as little as 5 min. The heat-induced injury involved not only the capacity to bind charged t-RNA but also the physical degradation of the ribosome as observed in changes in sedimentation profiles. As a standard, the

mesophile Candida utilis was subjected to similar treatment and was unaffected. Purohit and Finnerty (1967) have indicated that ribosomes as well as protein synthesizing enzymes are much more thermolabile in the psychophilic bacterium strain 82 than they are in the mesophile E. coli K-10.

The synthesis of RNA has also been implicated in the loss in viability of psychrophiles at moderately elevated temperatures. Harder and Veldkamp (1967) performed a continuous culture study of an obligately psychrophilic Pseudomonas species and measured the concentration of RNA in the cell as a function of temperature of incubation. It was found that the RNA concentration was lowest at 14°C, the optimum growth temperature of the organism. At lower temperatures the RNA content of the cells increased to compensate for the reduced reaction rates in the cell. Exposure of the cells to temperatures above the optimum but below the maximum growth temperature also resulted in increased levels of RNA. The authors stated that this increase in RNA probably counteracted an impairment of protein synthesis. Above the maximum growth temperature RNA synthesis ceased. Cessation of RNA synthesis was followed by cessation of protein synthesis. The investigators termed the increased synthesis of RNA above and below the optimum growth temperature "compensatory" synthesis. Further studies (Harder and Veldkamp 1968) showed that although the maximum growth temperature was 23°C respiration continued unimpaired after at least 5 hr of exposure to 25°C. Cells incubated at supermaximal temperatures showed a release of ultraviolet absorbing material and RNA synthesis could not be detected.

When substrate was added to cells at 14°C, the optimum growth temperature, a rapid increase in RNA was noted, followed by increases in DNA and protein. At 22.5°C no RNA synthesis could be detected but small increases in DNA content of the cells were noted. Under these conditions, no increase in protein was evident. At supermaximal temperatures a slight loss of cellular protein and DNA and a greater loss of RNA were observed. Harder and Veldkamp (1968) concluded that the inability of the organism to synthesize RNA at supermaximal temperatures is an important factor in fixing the maximum growth temperature. The study of thermo-sensitive RNA synthesis and degradation is not limited to psychrophilic organisms. Strange and Schon (1964) made similar observations with the mesophile Aerobacter aerogenes. Degradation of RNA occurred at temperatures slightly above the maximum growth temperature (36°C). Allwood and Russell (1968) studied Staphylococcus aureus exposed to supermaximal temperatures of 50°C and 60°C and observed several phenomena. A "diphasic" breakdown of intracellular RNA occurred at 50°C but not at 60°C. This suggested that the breakdown of RNA at 50°C may be due to enzymatic degradation. This view was shared by Malcolm (1968) who suggested that the release of RNA derivatives from the psychrophile M. cryophilus cell might be the result of the action of an enzyme (phosphodiesterase) active against RNA.

Attention to damage of cell membranes and subsequent leakage of intracellular components following exposure of psychrophiles to supermaximal temperatures has also been suggested as a mechanism of low temperature death in psychrophiles. Robinson and Morita (1966) showed a

correlation between loss in viability and leakage upon exposure of the psychrophilic bacterium to supermaximal temperatures. The material which leaked from the cell absorbed strongly in the ultraviolet region, i.e., 260 nM. This material was further characterized by Haight and Morita (1966) and was found to contain amino acids, proteins, nucleotides and polymeric RNA and DNA. The loss of 260 nM absorbing material from an obligately psychrophilic yeast was also reported by Nash and Sinclair (1968). Upon heating at temperatures from 15°C to 45°C, 260 nM absorbing material was released. The higher the temperature, the more rapidly the material was released. The 250/260 and 280/260 nM ratios indicated that the material probably consisted of nucleic acids or nucleic acid derivatives. Upon further investigation the leakage products proved to be amino acids and short polypeptides, inorganic phosphate and nucleotide monophosphate. They also determined by microscopic observation that leakage was not due to cell lysis. Harder and Veldkamp (1968) also reported leakage in their obligately psychrophilic Pseudomonas species. Hagen, Kushner, and Gibbons (1964) established that death preceded lysis in a gram-negative bacillus when heated to supermaximal temperatures. Malcolm (1968) stated that 5'-nucleotides of adenine, guanine, cytosine, and uracil leak into the medium for the first few hours of treatment, indicating some kind of selective leakage. However, polymeric RNA, DNA, and amino acids are released only after over 90% of the population are no longer viable. Only at this stage does the cell become permeable to a protein conjugating fluorescent dye. Malcolm stated that temperature-induced membrane damage and

leakage are not the cause of death but are a consequence of death. Allwood and Russell (1968) observed that the mesophile Staphylococcus aureus under heat stress leaked amino acids, protein, and 260 nM absorbing material. They measured the permeability of the cell to the fluorescent dye 8-anilino-1-naphthalene-sulfonic acid and concluded that membrane damage was at least partially responsible. They also showed that logarithmic phase cells released 260 nM absorbing material to a much larger degree than stationary phase cells. Clark, Witter, and Ordal (1968) observed that Streptococcus faecalis became more sensitive to salt upon heat treatment than did unheated cells. This also suggested a loss of selectivity of the membrane.

The evidence presented here indicates that while the sensitivity of psychrophiles to moderately elevated temperatures appears to be a consequence of heat sensitivity of cellular components it is difficult to ascertain any underlying unity in the mechanisms by which individual organisms are affected. The object of this study was to investigate the responses of three closely related and obligately psychrophilic yeasts to heat stress and to define in further detail the cellular response of Leucosporidium stokesii to heat stress. More specifically, studies of the synthesis of RNA, DNA, protein, and ATP as well as studies of the cell membrane and its role in the thermal death of an obligately psychrophilic yeast were undertaken.

MATERIALS AND METHODS

Organisms and Cultural Conditions

The organisms employed in this study were isolated in 1964 from samples of polar snow, ice, soil, seawater, and marine sediments from the arctic and antarctic regions (Sinclair and Stokes 1965). The organisms were designated Candida species P-7, P-8, P-16, and P-25. These organisms have recently been subjected to further taxonomic study and have been reclassified by Fell et al. (1969). Strain P-7 has been renamed Candida nivalis. Sexual stages of P-8 and P-16 have been observed and thus these organisms have been placed in a new genus, Leucosporidium. Strain P-8 has been renamed Leucosporidium frigidum; P-16 is now called Leucosporidium stokesii; P-25 is still designated as a Candida species. Each of these yeasts grows over the range of 0°C to 20°C. The optimum growth temperature is 15°C. Larkin and Stokes (1968) have reported growth of these yeasts below 0°C. Thus, this temperature is not the minimum growth temperature. Leucosporidium frigidum and Candida sp. P-25 are somewhat more sensitive to 25°C heat than are the other two yeasts, although all are quite sensitive to exposure to a temperature of 30°C (Sinclair and Stokes 1965).

Several media were employed in these studies. Growth was determined on a medium containing yeast extract, 10.0 g; glucose, 10.0 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; in one liter of glass distilled water. This medium was designated YAD medium and was solidified with 1.5% agar

as necessary. A second medium, labeled YNB medium, consisted of the following: Yeast Nitrogen Base (Difco), 6.7 g; KH_2PO_4, 1.0 g; glucose, 10.0 g; in one liter of glass distilled water. For radioactive tracer studies a medium (J-J medium) composed of the following was employed: yeast extract, 1.0 g; Yeast Nitrogen Base (Difco), 6.7 g; adenine sulfate, 0.01 g; thiamine, 0.01 g; uracil, 0.01 g; leucine, 0.01 g; thymine, 0.01 g; KH_2PO_4, 1.0 g; glucose, 10.0 g; in one liter of glass distilled water. This medium was modified from the YM-5 medium of Hartwell (1967).

After 48 hr incubation on a YAD slant, cells were suspended in 5 ml of sterile distilled water. One-half ml of this suspension was added to 50 ml of broth (YAD, YNB, or J-J) and incubated at 15°C on a New Brunswick reciprocating water bath shaker for 24 to 36 hr. This time period allowed the population to reach mid-logarithmic growth phase. Turbidity was measured with a Klett-Summerson photoelectric colorimeter, Model 800-3, using the red (no. 66) filter. A 1.0% (w/v) inoculum was transferred to the final flask (flask to medium volume ratio never exceeding 5:1) and incubated at 15°C for 36 hr to a turbidity of 100 Klett units.

Determination of Injury and Survival at Permissive and Restrictive Growth Temperatures

Cells were cultured in YNB medium to 100 Kletts and the culture was divided into three portions and incubated on a Burrell wrist-action shaker at 15°C, 25°C, and 30°C, respectively. At various time intervals samples were removed, diluted in double-distilled water containing

0.1% peptone, and immediately plated on complete (YAD) or basal (YNB) medium to determine viability and extent of injury. Those cells which were unable to form colonies on YNB but could form colonies on YAD were considered to be injured (Nash and Sinclair 1968). Those cells unable to form colonies on either YNB or YAD were defined as being dead.

Determination of Dry Weight and Turbidity Relationship

Cells were cultured to approximately 100 Klett units, harvested by centrifugation, washed in M/15 KH_2PO_4, and resuspended in double distilled water to different turbidities. Ten-ml samples in triplicate were transferred to previously weighed (dried to constant weight) planchets and dried at 80°C until no further weight change was noted. The triplicate samples were weighed on a Sartorius laboratory analytical balance and the appropriate calculations were made. In all subsequent experiments dry weight determinations were made by comparisons with the curves thus obtained.

Determination of Respiratory Activity

Respiratory activity was determined for *L. frigidum*, *L. stokesii* and *Candida* sp. P-25. Cells were grown to 100 Kletts (log phase), harvested by centrifugation and concentrated by a factor of five in M/15 KH_2PO_4 and placed in a refrigerated digital readout respirometer, Gilson Model GR-14, with and without exogenous glucose. After equilibration, readings were made at 5-min intervals until a constant rate had been established. Respiratory activity was measured at 25°C, the optimum temperature for activity of *L. stokesii* (Sinclair and Stokes 1965).

This optimum was shown to be valid for Candida sp. P-25 as well when respiratory activity was measured over the temperature range of 15°C to 35°C at five degree intervals. The effect of heat stress on respiration was determined with L. frigidum, L. stokesii, and C. sp. P-25 by observing respiratory activity after various periods of incubation in growth medium at supermaximal temperatures.

Determination of Leakage from the Cell after Exposure to Supermaximal Temperatures

In order to determine the extent of leakage of ultraviolet-absorbing material from the cell during heat injury, the cells were suspended in double distilled water during the heat treatment. In growth medium the leakage was masked by medium components. Samples were taken at intervals and optical density at 260 nM was measured. The optical density was determined in a Heath spectrophotometer, Model 701. These measurements were made on L. frigidum, L. stokesii and Candida sp. P-25. Further work on leakage of L. stokesii was carried out employing radioactive tracer techniques described below.

Ribonuclease Assay

Determination of the presence of RNAse (phosphodiesterase) in heat-stressed cells was undertaken by the technique outlined in the work of Koerner and Sinsheimer (1957). Cells were grown to mid-log phase on YNB medium and incubated at 30°C for 4 hr. Cells were harvested, washed and ground for 2 to 3 min in a chilled mortar and pestle with aluminum oxide, Sigma type 305, and finally suspended in 5 ml of

ice cold M/15 KH_2PO_4. The suspension was centrifuged at approximately 4000 x g for 10 min. The resulting supernatant was centrifuged at 10,000 x g for an additional 15 min and the supernatant was used for the assay. The supernatant was opalescent, indicative of the presence of protein. The following assay for the activity of RNAse was used. The reaction mixture consisted of 10 ml of 0.001 M Ca bis(p-nitrophenyl)-phosphate in 0.001 M ammonium acetate buffer, pH 8.8, and 6 ml of double distilled water. The reaction was initiated by the addition of 0.1 ml of the extract to 2.9 ml of the substrate in a cuvette at 25°C. Absorbancy at 440 nM was recorded at one-minute intervals. Potency of the extract was defined as the change in absorbance at 440 nM per minute divided by the absorbance of the reaction mixture at 280 nM times an appropriate dilution factor. Cell extracts for this determination were also prepared by the protamine sulfate method of Schlenk and Zydek-Cwick (1970).

Chemical Fractionation of the Cells of L. stokesii

Studies on the macromolecular composition of Leucosporidium stokesii were undertaken to determine the relationship between macromolecular synthesis and heat injury. Cells were grown to mid-log phase as described above, transferred to restrictive temperatures, 20°C and 30°C, and sampled at various times for determinations of RNA, DNA, and protein. Fractionation was carried out by two different techniques. The first was that described by Harder and Veldkamp (1968). Cells subjected to heat stress were concentrated by centrifugation. The pellets

were extracted with 2 ml of ice cold 0.2 N $HClO_4$ for 20 min, and the insoluble material was collected by centrifugation at 5000 x g in a refrigerated Sorvall RC-2B centrifuge. The sediment was extracted by a modified Schmidt-Tannhauser procedure (Munro and Fleck 1966). Four ml of 0.3 N KOH was added to the centrifuge tubes and the sediments were solubilized at 37°C for one hour. The system was cooled to 0°C, and 2.5 ml of 1.2 N $HClO_4$ was added; after standing for 10 min at 0°C the precipitate was collected by centrifugation as above. The sediments were washed with 2 ml of 0.2 N $HClO_4$, and RNA was determined in the pooled supernatants by the orcinol method described below (Schneider 1957). The DNA was extracted from the sediments as follows: 2.5 ml of 0.5 N $HClO_4$ was added to each tube and the suspension was heated to 70° C for 20 min. After centrifugation at 10,000 x g for 10 min, the extraction was repeated and the supernatants were pooled and tested for DNA by the diphenylamine test of Schneider (1957). The resulting sediments were dissolved in 2 ml of 1.0 N KOH and protein was determined by the technique of Lowry et al. (1951). These determinations will be summarized below.

The second method employed for the fractionation of the cells was that of Ogur and Rosen (in Hutchison and Munro 1961). Cells were initially extracted with 70% ethanol at 4°C for 30 min to remove soluble pools. The sediment was treated with 5 ml of a mixture of ethanol and ether (3:1) at 45°C for 30 min. The supernatants were decanted after centrifugation and the sediments were extracted with 5 ml of 0.1 N perchloric acid (PCA) for 30 min at 4°C. The supernatants from this

step constitute the nucleotide fraction. Sediments were treated with 1 N PCA for 18 hr at 4°C to solubilize the RNA. Centrifugation at 10,000 x g was employed to separate the sediments from the RNA-containing supernatants. The DNA was extracted with 1 N PCA for 30 min at 70°C. The remaining sediments were solubilized with NCS solubilizer and tested for protein.

Chemical Determinations

Standard chemical determinations of RNA, DNA, and protein were employed. The bioluminescence technique of Patterson, Brezonik, and Putnam (1970) was employed to determine the concentration of ATP.

The orcinol technique of Schneider (1957) was employed to determine the amount of RNA present. The extract to be tested was brought to a volume of 1.5 ml with distilled water and was added to the orcinol reagent, which contained 1 g of orcinol and 0.5 g of $FeCl_3$ in 100 ml of concentrated HCl. The reaction mixture was placed in a boiling water bath for 20 min. The resulting color was measured in the Heath spectrophotometer at 660 nM. Purified yeast RNA was used as a standard.

The amount of DNA present in the extracts was determined by the diphenylamine technique described by Schneider (1957). One gram of diphenylamine and 2.75 ml of reagent grade concentrated sulfuric acid were dissolved in 100 ml of reagent grade glacial acetic acid. This reagent was prepared fresh for each assay. Two ml of the diphenylamine reagent were added to one ml of the nucleic acid extract and the system was heated in a boiling water bath for 10 min. The reaction was read

in the spectrophotometer at 600 nM. Purified DNA (Sigma) was used as a standard.

Levels of ATP were determined using a modification of the method of Patterson et al. (1970). Lyophilized aqueous extracts of firefly lanterns were obtained commercially (Sigma Chemical Company) and stored in the freezer until ready for use. The firefly extracts also contain arsenate buffer. Each vial was rehydrated with 25 ml of double distilled water and stored in 5-ml lots in the freezer until use. Crystalline ATP (Sigma sodium salt) was used as the standard. Samples from the heat injury studies were pipetted into boiling 0.02 M tris buffer, pH 7.75, and incubated in a boiling water bath for 5 min. At the end of the incubation, the sample was filtered through a membrane filter, the remaining volume of the extract was recorded, and samples were frozen until assayed. Known concentrations of ATP carried through the extraction procedure showed no loss of activity. A liquid scintillation spectrometer (Packard Tri-carb Model 314-EX-2) was used to measure light emission. Gain was set at 53, voltage at 1000, and window from 0 to 1000. Background light emission from the luciferin-luciferase preparation was measured prior to each assay. One tenth of the prepared firefly extract (luciferin-luciferase preparation) was pipetted with an Eppendorf pipette into a Durham tube which had been installed in a normal scintillation-counting vial and the background light emission was measured. As the vial was taken into the counting chamber, 50 µl of the ATP-containing cell extracts were added with an Eppendorf pipette to insure accuracy. All extracts were added in the same time

relationship to counting. This is important because the luminescence decays exponentially with time. The intensity of the light produced is directly proportional to the amount of ATP present in the extract. Each sample was counted for one minute and the background was subtracted. The counts obtained were compared with those obtained with the ATP standards. This technique is highly sensitive and has been shown to be a reliable indicator of the levels of ATP in a population or of the biomass of a given system.

Uptake of Radioactive Precursors and Incorporation into Macromolecules

Uptake and incorporation of radioactive precursors were extensively employed in these studies; ^{14}C-L-leucine (UL), ^{14}C-L-arginine (UL), uracil-2-^{14}C, thymine-2-^{14}C, and thymine(methyl-^{3}H) were employed. In one set of experiments, cells were cultured in J-J medium containing the appropriate label, approximately 2.0 μc per ml, for 18 hr. These prelabeled cells were subjected to the heat treatment as described earlier and samples were collected at intervals. Cells were separated from the spent growth medium by filtration on Gelman membrane filters, pore size 0.45 microns, and the supernatants were counted directly without further treatment. Cells were treated with 70% ethanol at 4°C for 30 min and the ethanol fraction (pool fraction) was counted. Cells were then exposed to hot 1 N PCA (70°C) for 30 min and 0.1 ml of this supernatant (nucleic acid fraction) was counted in the Packard Tri-carb liquid scintillation counter (Model 3022), using settings suggested by the manufacturer for the particular isotope being used.

In a second series of experiments, cells were cultured in J-J medium and shifted to the restrictive temperatures (25°C or 30°C) at which time label was then added. As a positive control, label was also added to cells incubated at 15°C. During the course of heat stress, samples were removed at intervals and subjected to further analysis. Cells were separated from the surrounding medium by centrifugation or by filtration through Millipore filters (0.45 and 0.22 micron). One-tenth ml samples of the spent medium were added to scintillation vials and counted. Cell samples were treated as required in the individual experiments: some were directly counted (whole cell uptake experiments) and others were fractionated by previously described methods (incorporation experiments). In the case of fractionation, 0.1-ml samples were transferred to scintillation vials and counted. In the whole cell uptake experiments the cells from an entire 3.0 to 5.0-ml sample were added to the counting vial on the membrane used to collect them. In one case both ^{14}C and ^{3}H labels were employed at the same time. The fractionation and counting procedures were the same except that the scintillation counter was adjusted for dual-channel counting and the appropriate corrections were made.

Radioactive tracers were also employed to show the extent of damage to the cell membrane during heat stress and the ability of the cell to recover from brief periods of heat stress. Cells were shifted from 15°C to a restrictive temperature, i.e., 25°C, and incubated for 240 min. Samples were removed during this incubation at the following times: 0, 15, 30, 60, 120, and 240 min. Samples were shifted down to

15°C at each sampling interval. At the time of shift to 15°C, label was added (^{14}C-UL-L-leucine, 2.0 μc per ml). Each of these shift-down cultures was then sampled at 0, 30, 60, 120, and 240 min after the addition of label and whole cell uptake of the label was determined as described.

RESULTS

Viability of L. frigidum, L. stokesii, and Candida sp. P-25 Following Exposure to Supermaximal Temperatures

Percentage survival of three psychrophilic yeasts suspended in YNB medium and incubated at 15°C, 25°C, and 30°C for various periods of time is shown in Table 1. The data show that differences in heat sensitivity exist. Cells of all three yeasts showed no loss in viability when incubated at 15°C. At 25°C differences are evident. After 120 min of heat treatment viability of L. frigidum and Candida sp. P-25 decreased. In contrast, viability of L. stokesii increased. After 240 min the viability of all three yeasts decreased. At 30°C death occurred more rapidly. It was of interest to note that, although L. stokesii is less sensitive to 25°C heat, it is more sensitive to 30°C heat than either of the other two yeasts.

Dry Weight and Turbidity Relationship

Since the calculation of QO_2, the respiratory quotient, is based on the dry weight of cells used in the test and also to determine the size of the population for other experiments, it was deemed advisable to determine the relationship of dry weight to turbidity. The relationship between dry weight and turbidity for P-8, P-16, and P-25 is shown in Figure 1. For most experiments, the turbidity of the culture was adjusted to 100 Klett units. This is the equivalent of 1.3 mg dry

Table 1. Percent survival of three obligately psychrophilic yeasts upon exposure to supermaximal growth temperatures.*

Organism	Incubation temperature (°C)	Time of incubation (min) 0	120	240	480
P-8	15	100	100+	100+	100+
P-16	15	100	100+	100+	100+
P-25	15	100	100+	100+	100+
P-8	25	100	74	46	14
P-16	25	100	100+	91	56
P-25	25	100	95	88	47
P-8	30	100	32	6.5	0.3
P-16	30	100	13	2.5	0.3
P-25	30	100	26	3.7	0.1

*Injury carried out in YNB medium.

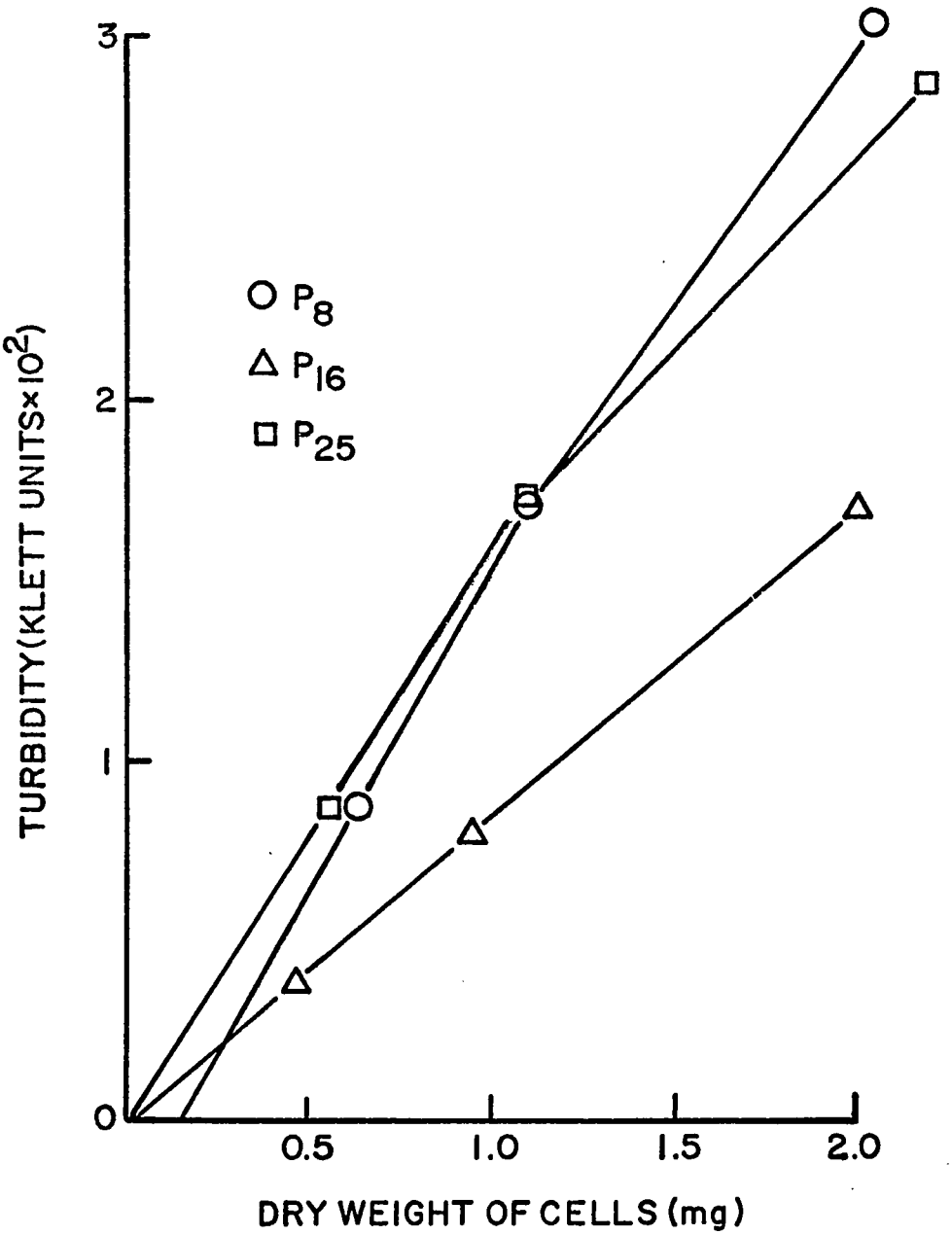

Figure 1. Dry weight and turbidity relationship of L. frigidum, L. stokesii (P-16), and Candida sp. P-25.

weight cells/ml. All determinations of the dry weight-turbidity relationship were carried out on log phase cells.

Respiratory Activity of L. stokesii, L. frigidum, and Candida sp. P-25 Following Exposure to Supermaximal Temperatures

Respiratory activity of whole cell suspensions of the three yeasts was tested after 2 hr of exposure to restrictive temperatures. The cells were suspended in YNB medium during heat stress. The data are presented in Table 2. Leucosporidium frigidum (P-8) lost 69% of its respiratory activity but only 26% of its viability at 25°C while at 30°C L. frigidum lost all of its respiratory activity and retained 32% viability after 2 hr incubation. L. stokesii lost only one-half of its respiratory activity while the viability declined by over 85%. Respiratory activity of L. stokesii is much less sensitive to heat stress than the respiratory activity of the other two yeasts; L. stokesii does not lose respiratory activity nearly as quickly as it loses viability when exposed to supermaximal temperatures. The differences in response of respiratory activity to thermal stress in these organisms implies that different mechanisms of thermal death may be operating in each of the three psychrophilic yeasts.

Leakage of Ultraviolet Absorbing Material Following Exposure of L. frigidum, L. stokesii, and Candida sp. P-25 to Supermaximal Growth Temperatures

Leakage of OD_{260} absorbing material from log phase cells of L. frigidum (P-8), L. stokesii (P-16), and Candida sp. P-25 is shown in Table 3. Cells were incubated in M/15 KH_2PO_4 at 15°C, 25°C and 30°C.

Table 2. Effect of incubation at supermaximal temperatures on the respiratory activity (QO_2) of P-8, P-16, and P-25.*

Organism	Incubation temperature (°C)	Time of incubation		Percent viability
		0 time (QO_2)	120 min (QO_2)	
P-8	15	134	102	100
P-16	15	63	50	100
P-25	15	51	72	100
P-8	25	134	42	74
P-25	25	51	0	95
P-8	30	134	0	32
P-16	30	63	30	13
P-25	30	51	0	26

*QO_2 is defined as microliters of oxygen taken up/mg dry weight/hour.

Table 3. Cellular leakage (OD_{260}) of P-8, P-16, and P-25 at permissive and restrictive growth temperatures.*

Organism	Incubation temperature (°C)	Time of incubation (min) 0	120	240	480
P-8	15	0	-	-	0.102
P-16	15	0	0	0	0.014
P-25	15	0	-	-	0.000
P-8	25	0	0.069	0.140	0.208
P-16	25	0	0	0.061	0.130
P-25	25	0	0	0.051	0.070
P-8	30	0	0.253	0.307	0.367
P-16	30	0	0.182	0.240	0.321
P-25	30	0	0.130	0.383	0.710

*Heat injury carried out in M/15 KH_2PO_4.

Five-ml samples were removed after 2, 4, and 8 hr of exposure to temperatures of 15°C, 25°C, and 30°C. Cells were removed by centrifugation and the OD_{260} was determined directly on the Heath 701 spectrophotometer. At 15°C, the optimum growth temperature, none of the cultures show appreciable leakage over the entire period of the experiment. At 25°C, leakage of 260 nM absorbing material from L. frigidum occurs sooner and to a greater extent than in either of the other yeasts. At 30°C, leakage of 260 nM absorbing material occurs more rapidly and is evident in all three yeasts after 2 hr of incubation. Candida sp. P-25 shows the greatest leakage of all the organisms tested. Leucosporidium frigidum shows the next largest degree of leakage and L. stokesii shows the least leakage of the three organisms at a stressing temperature of 30°C. Thus the three organisms differ with respect to heat-induced leakage.

Determination of Thermal Death and Injury in L. stokesii (P-16) Following Exposure to Supermaximal Temperatures

Leucosporidium stokesii has been studied extensively (Sinclair and Stokes 1965; Grant, Sinclair, and Nash 1968; Nash, Grant, and Sinclair 1969; Nash and Grant 1969). These studies all employed temperatures at least ten degrees above the maximum growth temperature (20°C). In addition, previous study has shown that the optimum temperature for fermentation and respiration of L. stokesii is 25°C (Sinclair and Stokes 1965) and that protein synthesis was little affected by 25°C heat (Nash, Grant, and Sinclair 1969). Viability of L. stokesii at 15°C, the optimum growth temperature, 25°C, five degrees above the

maximum growth temperature, and 30°C was determined. Cells were cultured to 100 Kletts, mid-log phase, harvested, resuspended in fresh YNB medium, and exposed to temperatures of 15°C, 25°C, and 30°C for 8 hr. Samples were removed, diluted, and plated on YNB medium. The data are presented in Figure 2. The rate of growth at 15°C is exponential as expected. At 25°C, viability increases over the first 2 hr of treatment and then decreases logarithmically. At 30°C, viability decreases markedly. After as little as 2 hr incubation only 13% of the initial viability remains. The leakage data shown in Table 3 correlate well with the viability data.

When the cells were plated on both YNB and YAD media, the degree of injury to the population could be directly determined. The YAD is a nutritionally rich medium containing yeast extract, while YNB medium is a chemically defined minimal medium which supports the growth of uninjured L. stokesii. Injured cells are defined as cells that will form colonies on YAD medium but not on the minimal YNB medium. Percentage death was determined by comparison of initial YAD counts and YAD counts after various periods of heat stress. Percentage injury of log phase cells of L. stokesii is shown in Table 4. At both 30°C and 35°C, percentage injury is highest after 30 min of heat treatment and decreases over the remaining 30-min period. As the length of the heat treatment increases, the extent of death increases. These data suggest that the injured cells are dying at a rate greater than they are being produced.

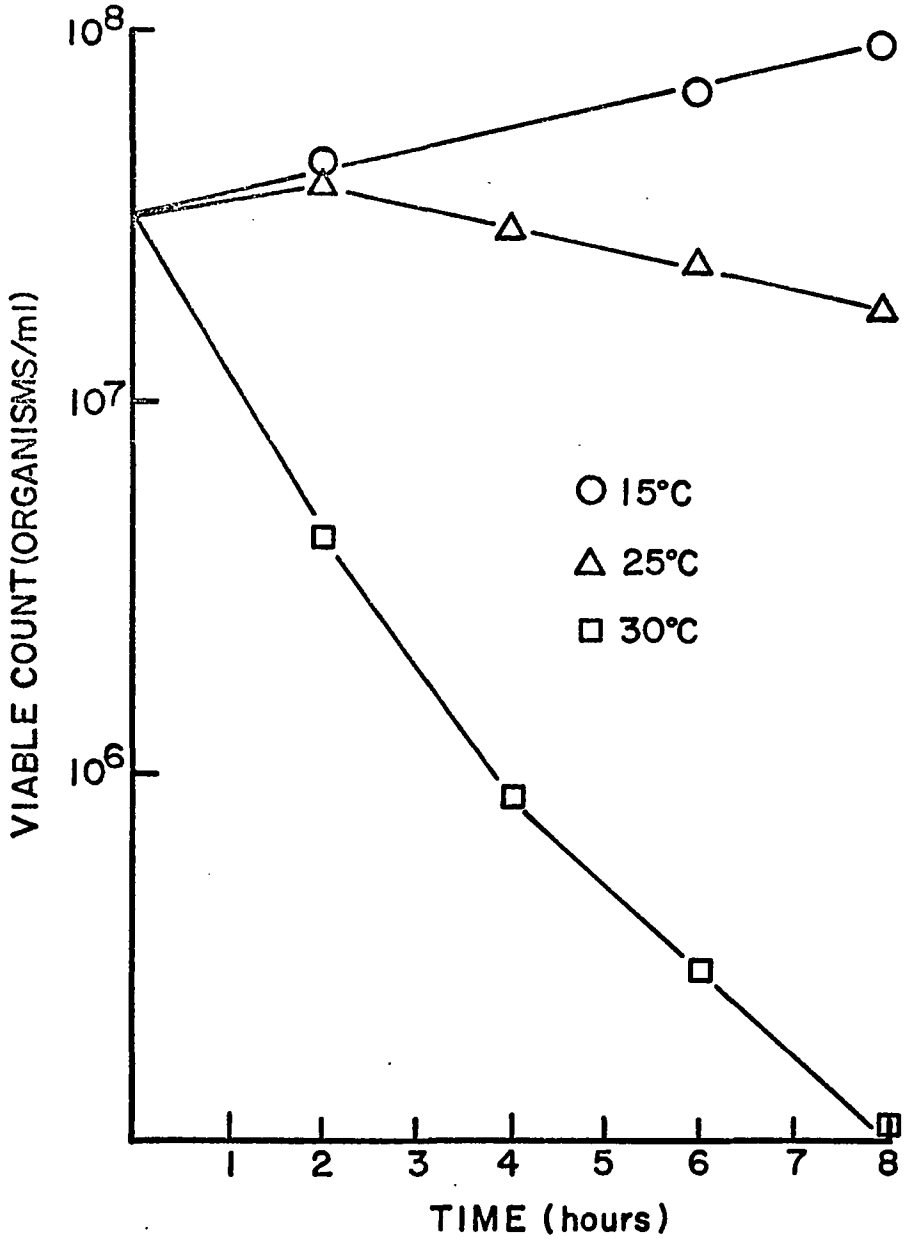

Figure 2. The effect of supermaximal temperatures on viability of Leucosporidium stokesii suspended in YNB medium.

Table 4. Injury and death of Leucosporidium stokesii exposed to 30°C and 35°C heat.

Temperature of incubation	Time of treatment (min)	YAD	YNB	Percent injury	Percent death
30°C	0	24.2	26.0	0	0
	30	15.0	1.61	89	38
	60	3.6	0.93	74	85
35°C	0	24.2	26.0	0	0
	30	1.11	0.33	70	95
	60	0.14	0.09	34	99

Cellular Leakage of L. stokesii Following Exposure to Restrictive Temperatures

It has been suggested (Malcolm 1968) that leakage of OD_{260} absorbing material from cells in response to heat stress might be caused by the enzymatic degradation of intracellular RNA. Moreover, the 260 nM absorbing material leaking from the yeast Candida nivalis (P-7) was identified as 5'-uracil monophosphate (Nash and Sinclair 1968). As indicated (Table 3) optical leakage from L. stokesii also occurs at 25°C and 30°C. In order to determine the source of the leakage the following preliminary experiment was carried out. Cells were prelabeled with ^{14}C-uracil for 8 hr in J-J medium. Following incubation the cells were removed by centrifugation from the medium, suspended in M/15 KH_2PO_4, and incubated at 15°C and 30°C. At various time intervals samples were removed, the suspensions were filtered, and the supernatants and cell sediments were examined for radioactivity. No loss of radioactivity to the medium was observed at 15°C while at 30°C the counts in the supernatant increased from approximately 7,500 counts per min per ml of culture to over 10,000 cpm/ml. Concomitantly, counts in the cells decreased by more than 10%. From these data it was inferred that leakage of uracil derived material occurred from L. stokesii following heat shock. These data are in agreement with those of Nash and Sinclair (1968) for the related yeast C. nivalis (P-7). In order to determine whether the leakage was due to the breakdown of intracellular RNA, the following experiment was performed. Cells were prelabeled for 18 hr in J-J medium containing 0.2 µc/ml ^{14}C-uracil. Half of the culture was transferred to nonradioactive medium for 2 hr in order to reduce the level

of radioactivity in the pools of the cells. Samples were removed, cells were collected on membranes, separated into two fractions by treatment with 70% ethanol to remove the soluble pools, and treated with hot 1 N PCA for 30 min to remove the nucleic acids. The two fractions thus obtained were transferred to counting vials and the radioactivity of each sample was determined. The data are shown in Figure 3. The 15°C and 30°C nucleic acid fractions retained all of the radioactivity initially present at 0 time. At 25°C a small, i.e., 10%, decrease occurred. In contrast, exposure of cells of L. stokesii to 25°C and 30°C heat stress resulted in a marked loss of radioactivity from the respective pool fractions. At 25°C, for example, less than 50% of initial activity remained after 2 hr of heating. After 4 hr of heating less than 30% of the initial activity remained. Similar results were observed for 30°C heated cells. In this case, however, leakage was more extensive. After 4 hr of heating, less than 5% of the initial radioactivity remained in the pool fraction. These data imply that breakdown of intracellular RNA is not the primary source of leakage in L. stokesii. In the case of the chased culture no radioactive leakage was noted at 25°C, and only a slight amount of leakage occurred at 30°C even after 2 hr of incubation at the restrictive temperature.

In order to eliminate the possibility of enzymatic degradation of RNA in L. stokesii during heat stress, the following experiment was performed. Cells were cultured to mid-log phase and were subjected to heat stress as described above. Samples were removed and cell-free extracts were prepared by alumina grinding and protamine sulfate

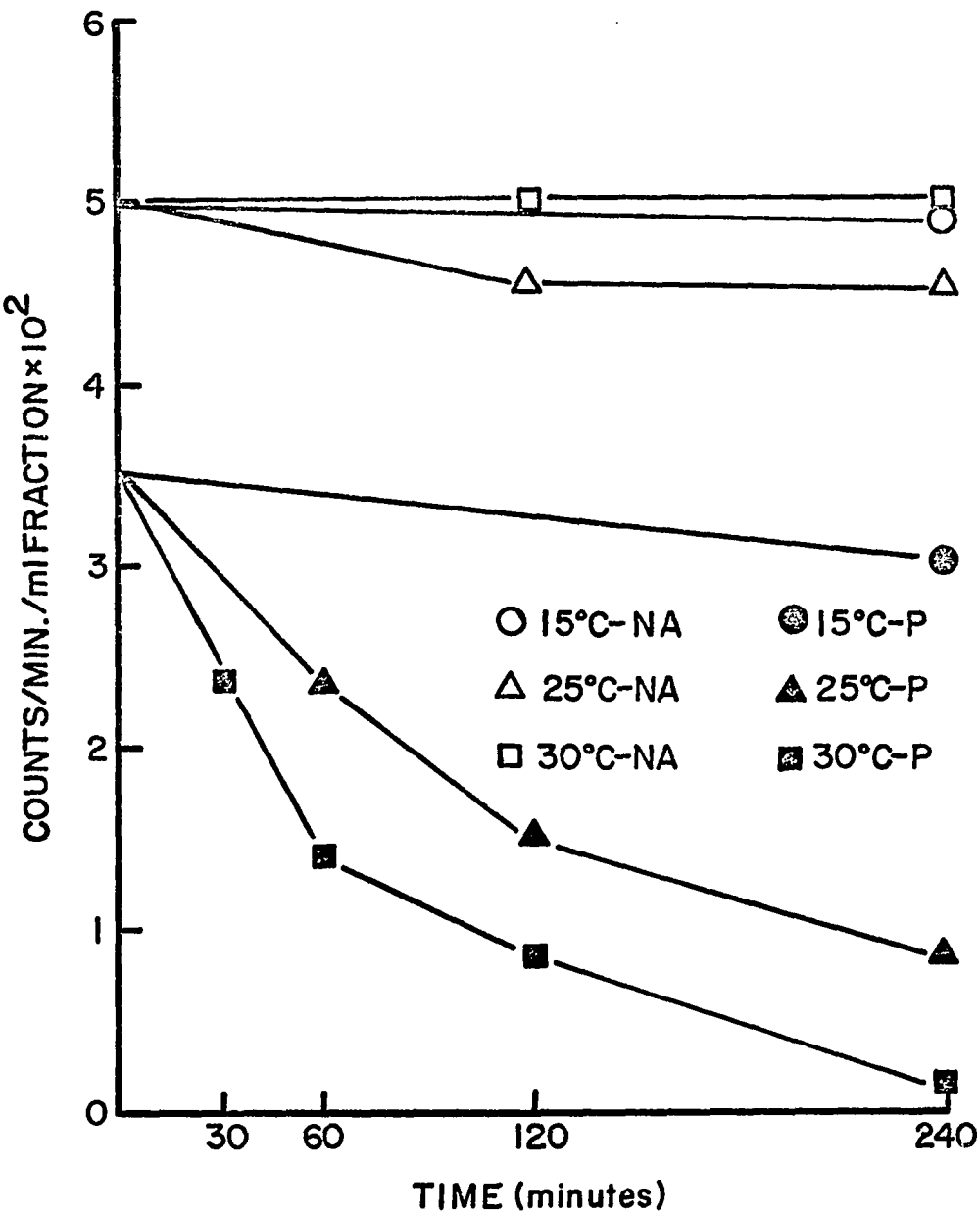

Figure 3. Leakage of radioactive uracil from cells of L. stokesii upon exposure to supermaximal temperatures.

treatment. The extracts were tested for RNAse activity. All attempts to demonstrate the enzyme were unsuccessful. These data suggest that active degradation of intracellular RNA and subsequent leakage of products are not primary factors in the determination of the maximum growth temperature of L. stokesii.

The Effect of Permissive and Restrictive Growth Temperatures on the Uptake of Radioactive Precursors by Whole Cells of L. stokesii

The uptake of radioactive precursors into various macromolecules by whole cells of L. stokesii was examined in cultures incubated at permissive and restrictive growth temperatures. Cells were incubated in J-J medium containing 0.2 μc/ml of ^{14}C-uracil, ^{14}C-thymine, ^{3}H-thymine, ^{14}C-leucine, and ^{14}C-arginine alone and in combination as indicated. Cells were heat-stressed for varying periods of time, samples were removed during the heat stress, whole cells were collected on membrane filters, and counts were made to determine the amount of label taken up by the cells. The data are shown in Figures 4, 5, and 6. Leucine uptake is rapid and essentially complete at 15°C with 88.3% of the label appearing inside the cell in 90 min (Figure 4). At 25°C, uptake is markedly reduced. Uptake of leucine occurs during the first hour of the heat stress. After 2 hr, however, no radioactivity could be detected in the cells incubated at 30°C. Heat treatment has a definite and profound effect on the uptake of leucine by the cells of L. stokesii. The uptake of labeled thymine (Figure 5) is initially stimulated at both of the restrictive temperatures as compared with the 15°C control culture. At 25°C, uptake occurs for the first hour followed by

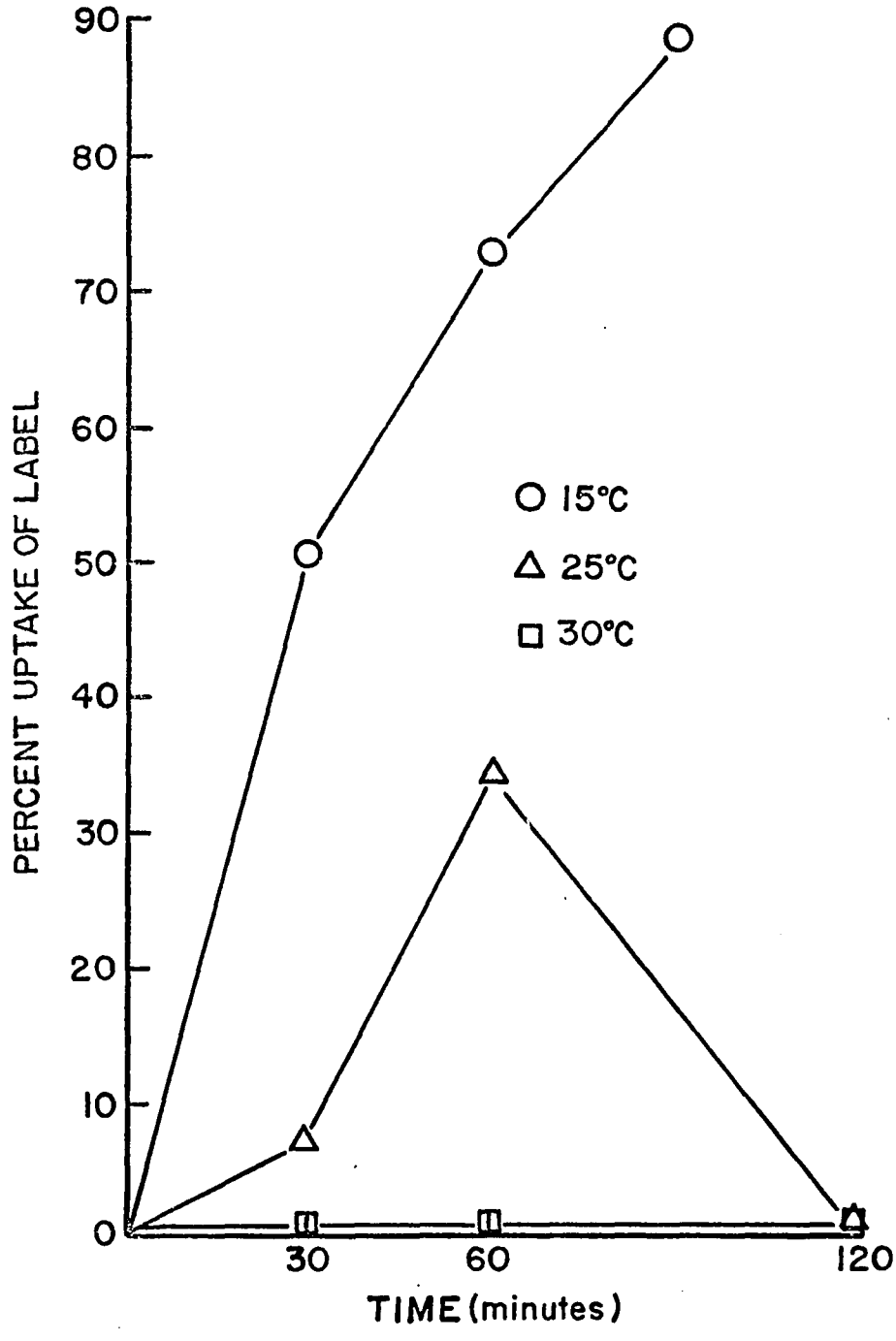

Figure 4. The effect of permissive and restricture growth temperatures on the uptake of radioactive leucine by whole cells on L. stokesii.

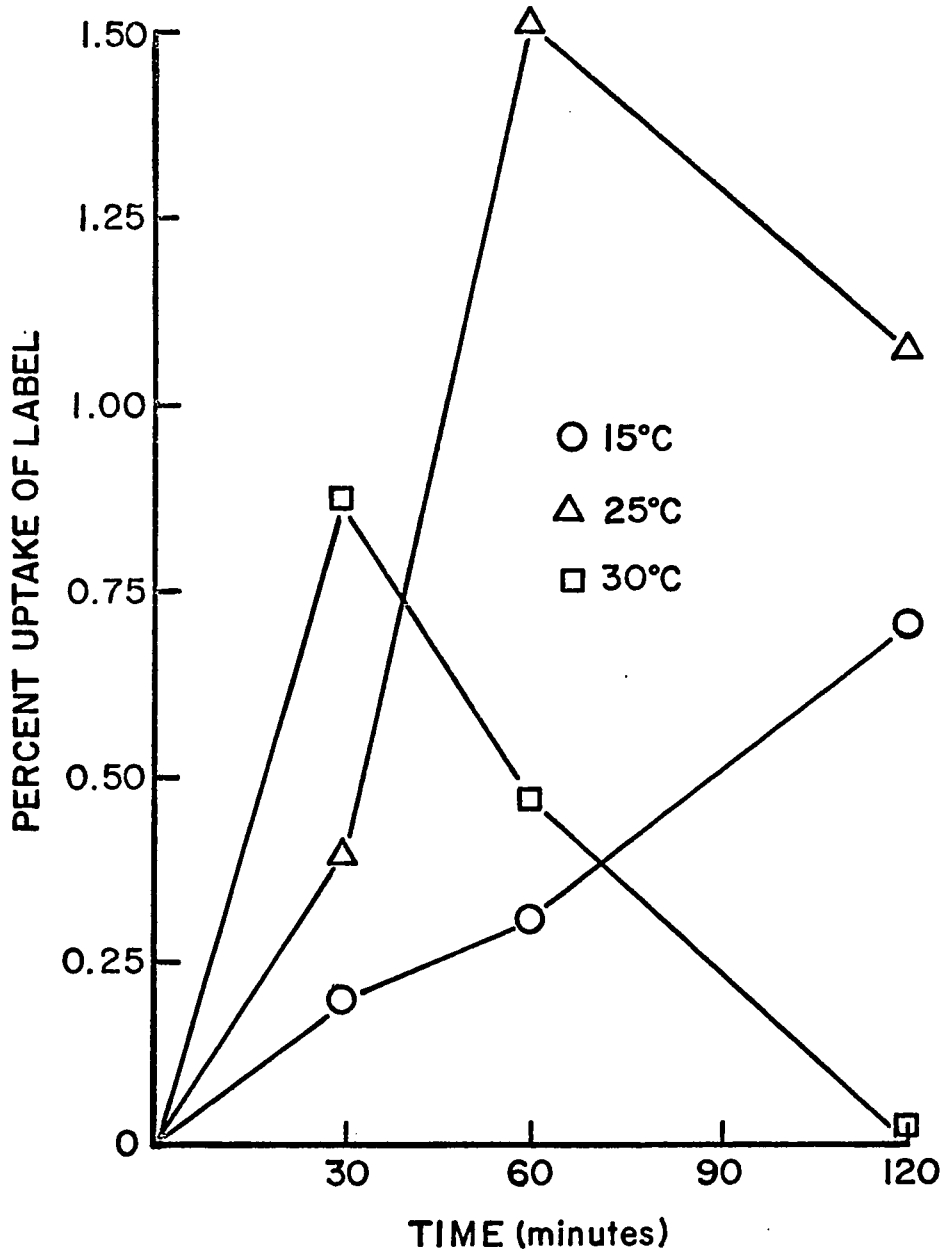

Figure 5. The effect of permissive and restrictive growth temperatures on the uptake of radioactive thymine by whole cells of L. stokesii.

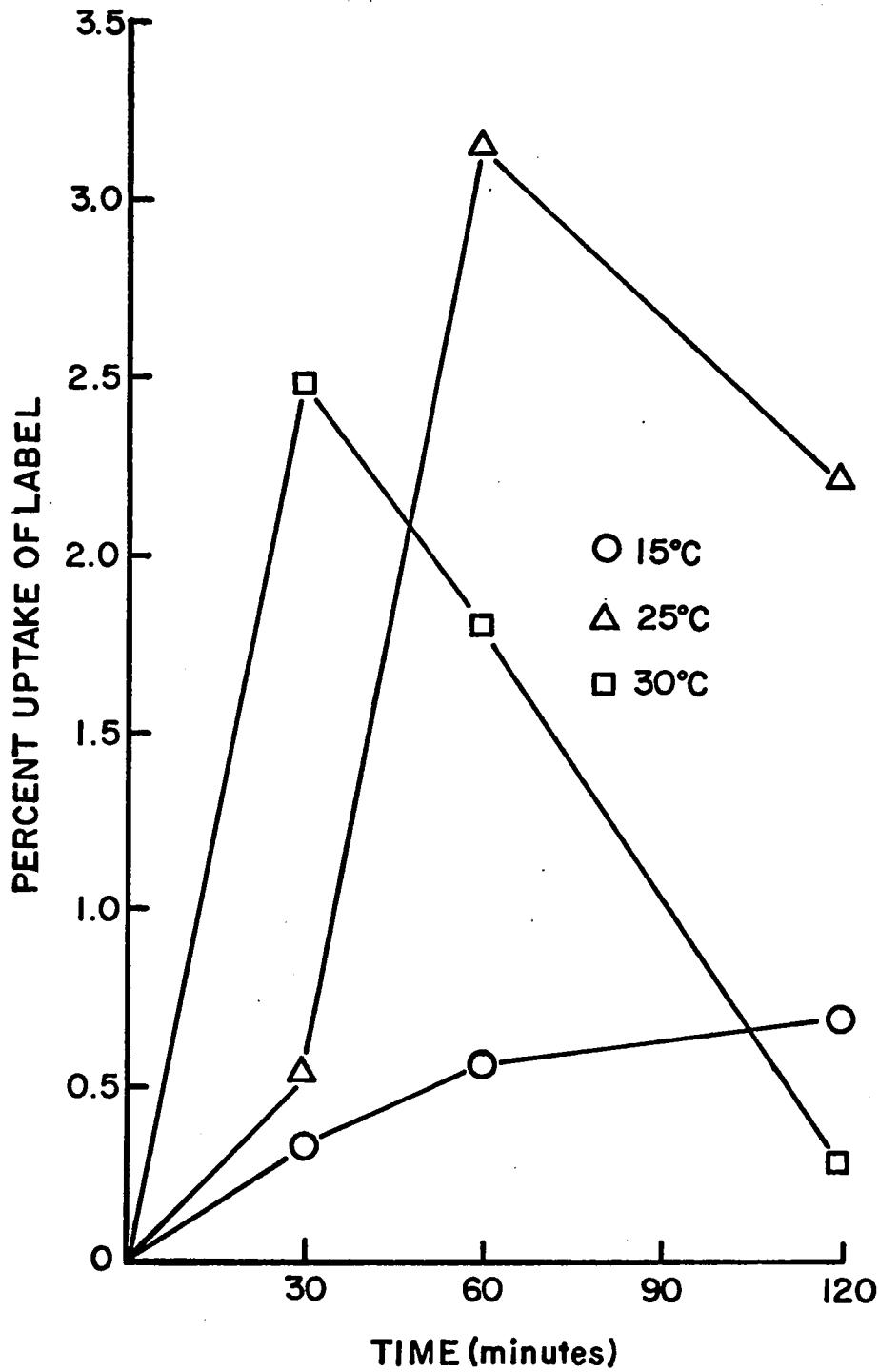

Figure 6. The effect of permissive and restrictive growth temperatures on the uptake of radioactive uracil by whole cells of L. stokesii.

release of radioactivity back into the medium. At 30°C, uptake occurs only for 30 min followed by release from the cell. In the control culture an orderly uptake of thymine is evident. The pattern of whole cell uptake of radioactive uracil (Figure 6) is much the same as that of thymine. At 15°C an orderly uptake is noted; at 25°C initial stimulation of uptake was observed for the first 2 hr of the treatment followed by leakage of radioactivity into the medium. Uptake of uracil occurs at 30°C for 30 min followed by rapid leakage from the cell. Only 0.28% of the label remained in the cell after 2 hr of the treatment. The implications of the stimulation of uptake of uracil and thymine and the sharp decrease in the uptake of leucine will be discussed below.

Reversibility of Heat-induced Membrane Damage by Incubation of *L. stokesii* at Permissive Growth Temperatures

Cells were cultured as described in J-J medium at 15°C to 100 Kletts and were then shifted, in growth medium, to 25°C for 4 hr. During this incubation period samples were removed at the following times: 0, 15, 30, 60, 120, and 240 min. These samples were placed at 15°C and ^{14}C-leucine was added. The uptake of leucine by each of the cultures shifted to 15°C was determined as described in the preceding section. The results are shown in Figure 7. If heat-induced membrane damage was irreversible, then the uptake patterns for the injured, shift-down, 15°C cultures should differ from the uptake pattern of the nonstressed 15°C control. The data (Figure 7) indicate no difference in the

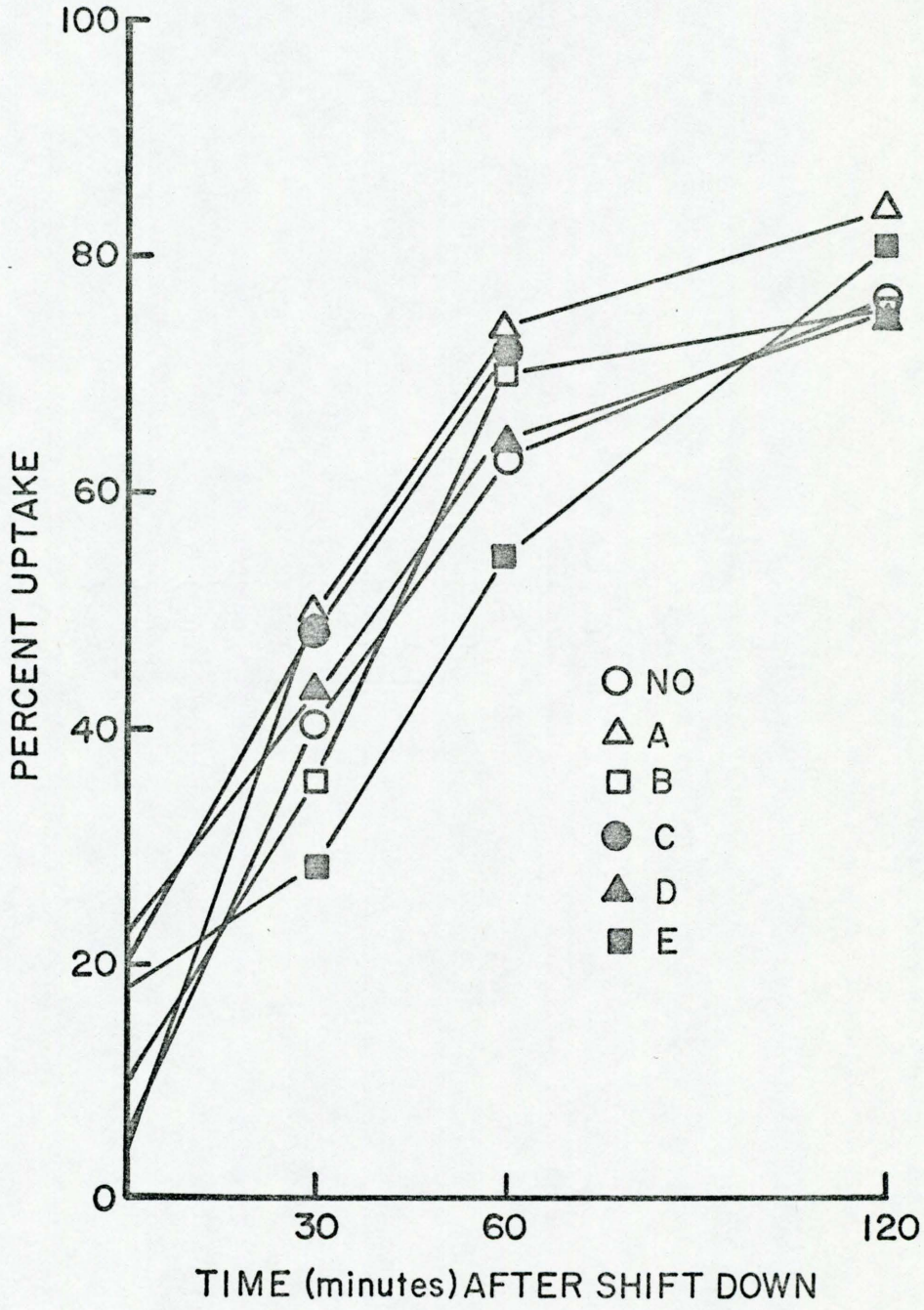

Figure 7. Whole cell uptake of radioactive leucine by cells of L. stokesii which had been exposed to 25°C and replaced at the permissive growth temperature after various time intervals.

pattern of leucine uptake irrespective of whether the cells were stressed or not. Thus any damage to the cell membrane at 25°C appears to be almost instantaneously reversible if indeed damage does occur.

Protein Synthesis and Heat Injury in L. stokesii

Chemical Determinations of Protein Synthesis of L. stokesii Following Heat Stress

Cells were cultured to 100 Kletts in YNB medium at 15°C and shifted to the restrictive temperatures, 25°C and 30°C. Samples were removed at various intervals and protein content was quantitatively determined. The method of Lowry et al. (1951) was employed for these determinations. The data are shown in Figure 8. At 15°C the amount of protein in the sample increased over the entire incubation period. This was also the case at 25°C although the levels of protein detected were smaller than in the 15°C control culture. At 30°C the amount of protein was higher than the 15°C and 25°C cultures after 30 min incubation. The level of protein fell after 30 min of treatment at 30°C and decreased over the remaining 210 min of the incubation period. These data correlate well with data of Nash, Grant, and Sinclair (1969) in that the protein synthetic apparatus of L. stokesii has been shown to be heat-sensitive to 35°C heat. These authors also noted that incorporation of amino acids into proteins by a cell-free system was little affected by incubation at 25°C. The data reported here indicate that at 25°C protein is being produced throughout the period of stress and

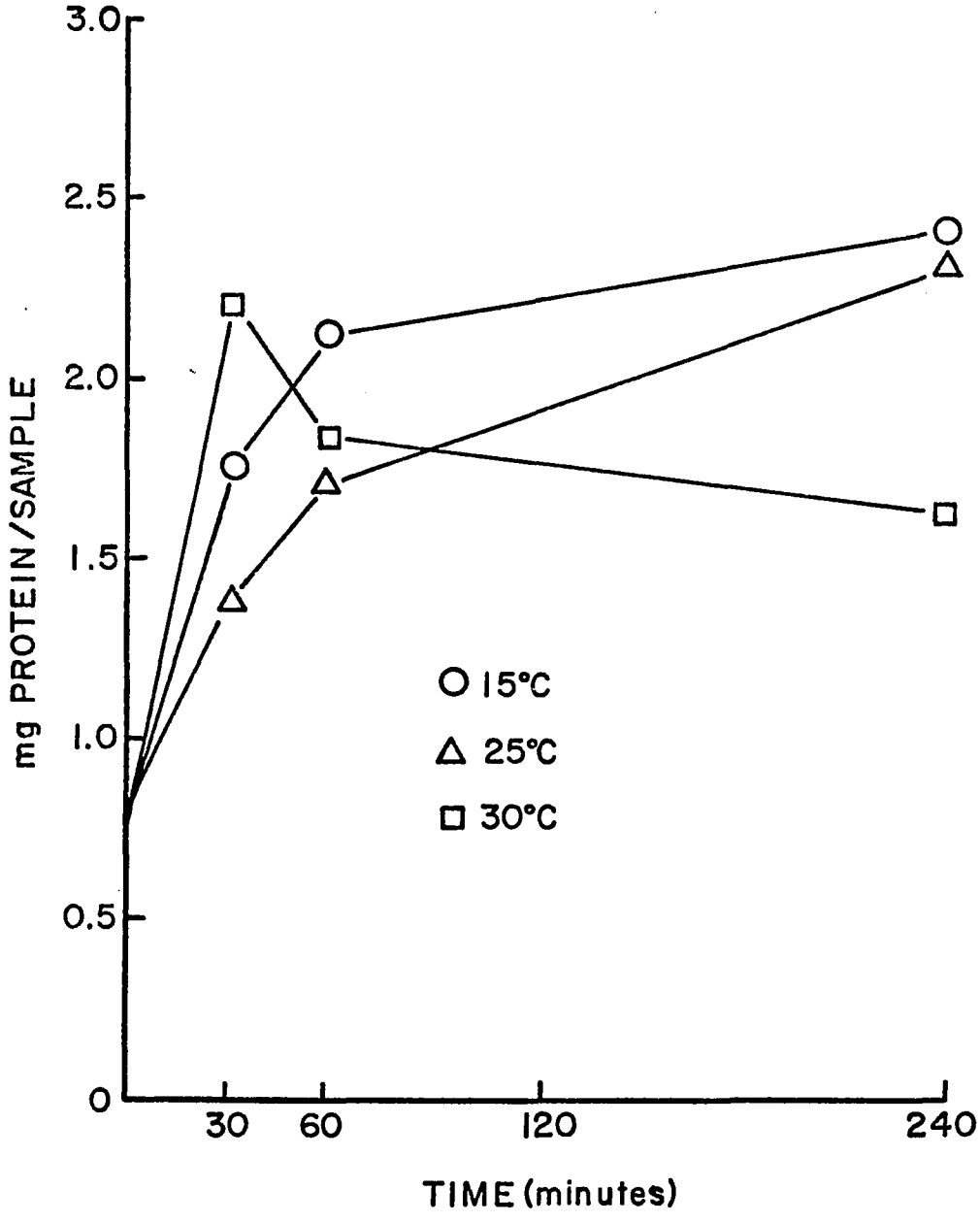

Figure 8. The effect of permissive and restrictive growth temperatures on the protein content of L. stokesii.

thus protein synthesis is not directly involved in the determination of the maximal growth temperature of L. stokesii.

Radiotracer Determinations of Protein Synthesis

Leucosporidium stokesii was cultured in J-J medium containing ^{14}C-leucine (0.2 μc/ml) at 15°C and 30°C for one hour. Cells were collected by centrifugation at 4000 x g in the cold and fractionated by the Ogur-Rosen method. Percentage recovered label in each fraction was calculated. The results are shown in Figure 9. At 15°C less than 3.0% of the recovered label was found in the pool fraction. Approximately 94% of the label was recovered from the nucleic acid and protein fractions. At 15°C protein synthesis was unimpaired. At 30°C however 88% of the label was recovered from the pool fraction and only 9.8% from the nucleic acid and protein fractions. Incorporation of leucine into protein is markedly impaired at 30°C. These results support those of Nash et al. (1969).

The chemical data indicated that the cell produced protein during the course of heat stress at 25°C and for the first 30 min at 30°C. The tracer data above indicated that no leucine is incorporated at 30°C. In order to resolve this difference the following experiment was carried out. Cells were cultured to 100 Kletts at 15°C in J-J medium, separated into four portions and incubated at 25°C and 30°C in the presence of either ^{14}C-arginine or ^{14}C-leucine. Arginine was selected since Nash et al. (1969) showed that although the leucyl transferase was sensitive to 30°C heat, the arginyl transferase was not sensitive.

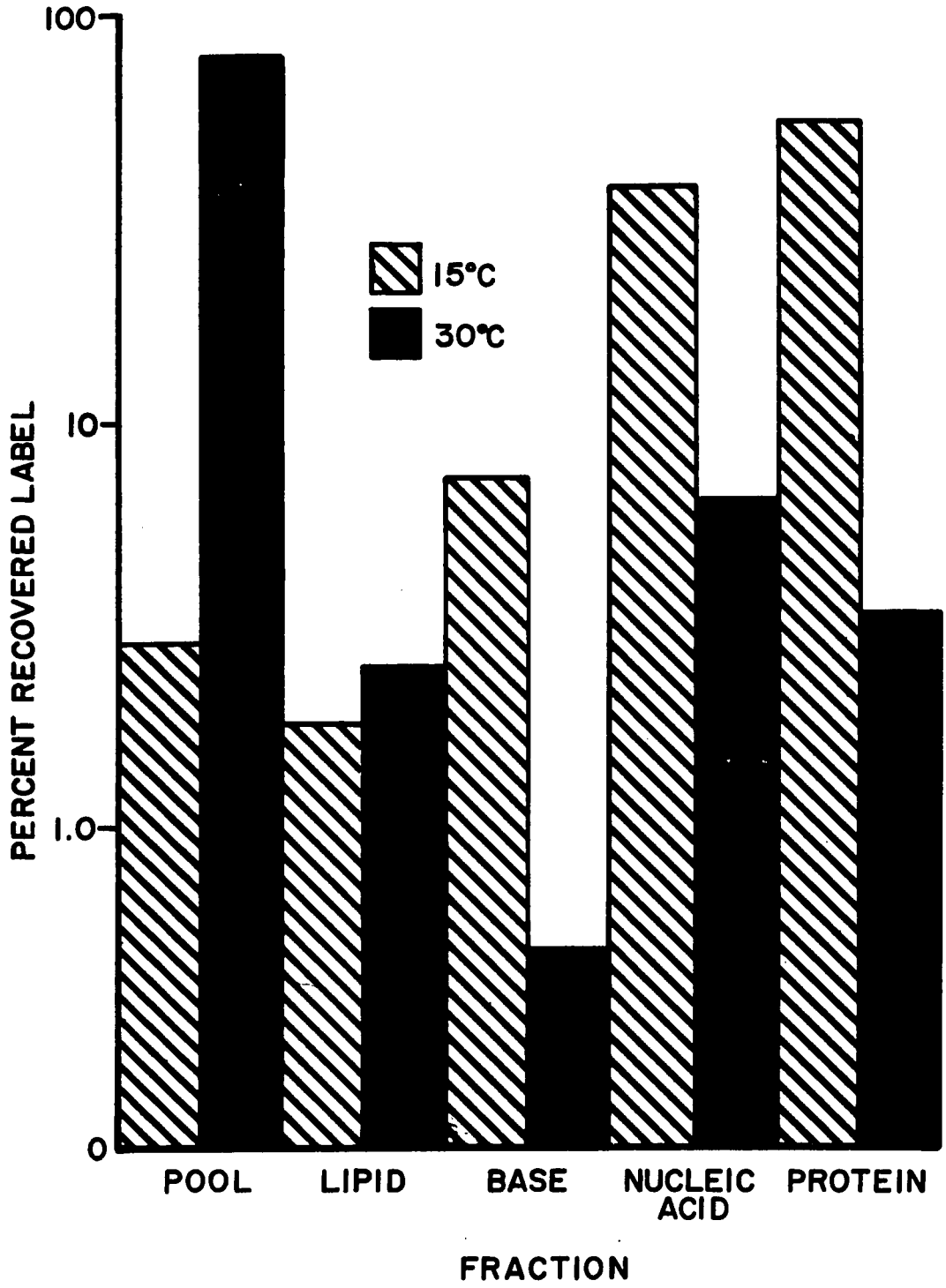

Figure 9. The effect of permissive and restrictive growth temperatures on the incorporation of radioactive leucine into cells of L. stokesii.

Samples were removed at 0, 15, 30, 60, and 120 min and the cells were extracted with hot 1 N PCA for 30 min. The radioactivity of the protein extract was determined. The results are shown in Figure 10. At 25°C both arginine and leucine are incorporated into the protein fraction of the cell. At 30°C leucine is not incorporated, as expected. A small amount of arginine, however, was incorporated into the protein fraction during the first 30 min of incubation. These data agree in some degree with the chemical data reported (Figure 7) in that protein is produced for the first 30 min of heat treatment at 30°C. The sensitivity of incorporation of leucine implies that the protein being produced may be deficient in leucine and may therefore be nonfunctional Thus protein synthesis occurs at 25°C and may occur for a limited period even at 30°C. This implies that a lesion in the protein synthetic apparatus is not primarily responsible for thermal death at 25°C.

Nucleic Acid Synthesis and Heat Injury in L. stokesii

Chemical Determinations of Nucleic Acid in L. stokesii During Heat Stress

Techniques similar to those used for the determination of protein synthesis were employed for chemical determinations of nucleic acid synthesis. Cells were cultured to 100 Kletts in J-J medium at 15°C, the optimum growth temperature and shifted to 25°C and 30°C for 240 min. Samples were removed, the cells collected by centrifugation, fractionated by the Ogur-Rosen method, and the amounts of RNA and DNA were determined by the techniques of Schneider (1957). The data from

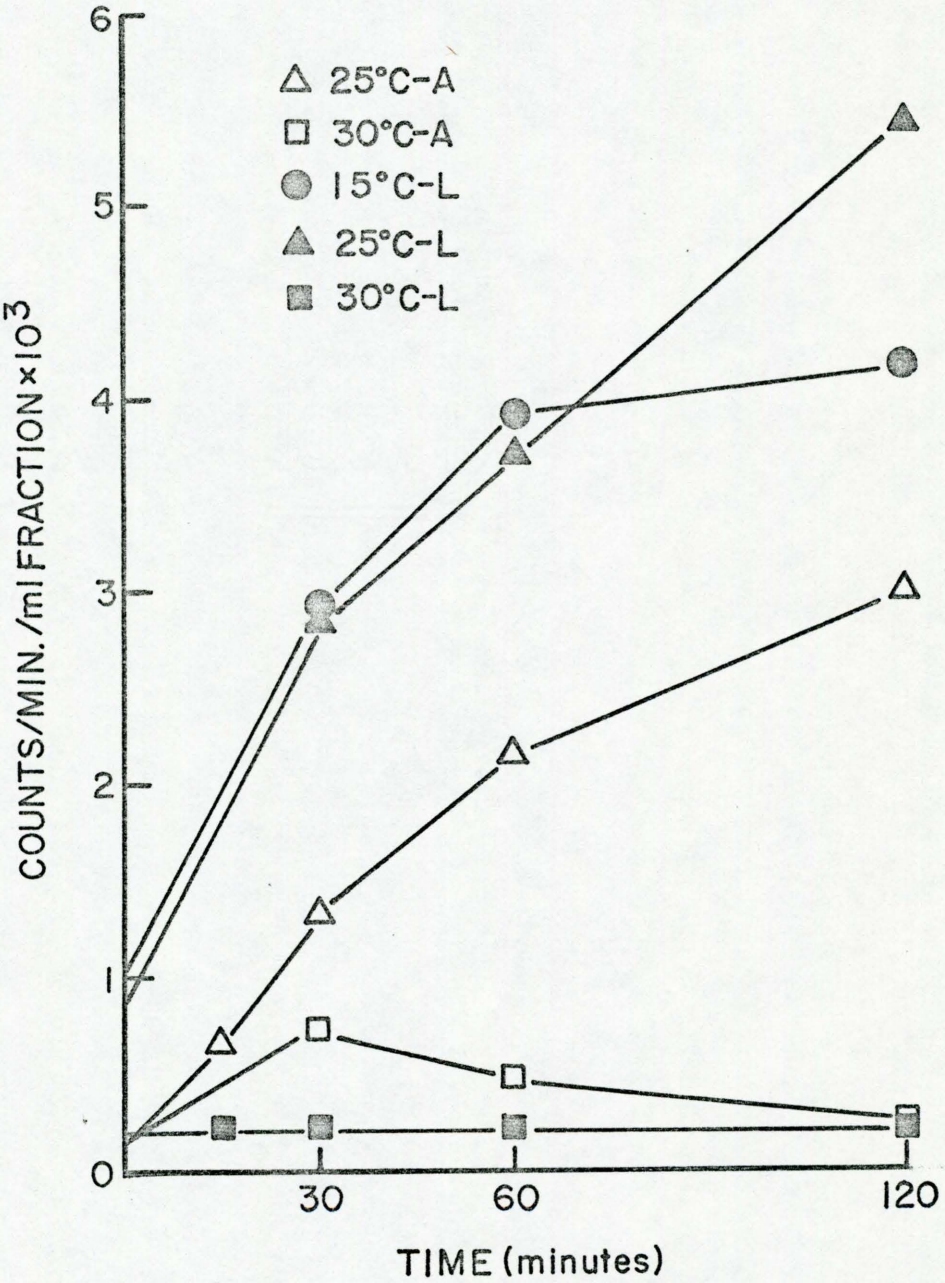

Figure 10. The effect of permissive and restrictive growth temperatures on the incorporation of radioactive leucine and radioactive arginine into hot 1 N PCA insoluble material of L. stokesii.

these determinations are shown in Figures 11 and 12. The DNA content of the cells exposed to various temperatures is shown in Figure 11. At 15°C the amount of DNA approximately doubles over the 4-hr incubation period. A slight increase in the levels of DNA for the first 30 min of incubation at 25°C and 30°C was noted. The DNA content of the cells remains relatively constant over the remaining 210 min of incubation.

The RNA content of cells incubated at 15°C, 25°C, and 30°C for 240 min is shown in Figure 12. At 15°C the amount of RNA in the culture increased over the entire incubation period. At 25°C an increase in RNA content is evident for the first 30 min of heat treatment. The level of RNA then declined over the remaining incubation period. At 30°C, however, the amount of RNA in the culture decreased from time zero with no initial stimulation of RNA production.

Incorporation of Radioactive Precursors into the Nucleic Acids of *L. stokesii* at Permissive and Restrictive Growth Temperatures

Cells were cultured to 100 Kletts in YNB medium at 15°C and shifted to 30°C for one hour; ^{14}C-uracil or ^{14}C-thymine (0.2 μc/ml) were added at the time of shift. Cells were collected by centrifugation and fractionated. Five major fractions including the pool fraction, lipid fraction, base fraction, nucleic acid fraction, and protein fraction were obtained. Percentage uptake of label into each fraction was calculated. The results of these determinations are presented in Figures 13 and 14. In the case of thymine incorporation (Figure 13) over twice as much label was found in the nucleic acid fraction at 15°C as was found in the same fraction at 30°C. The

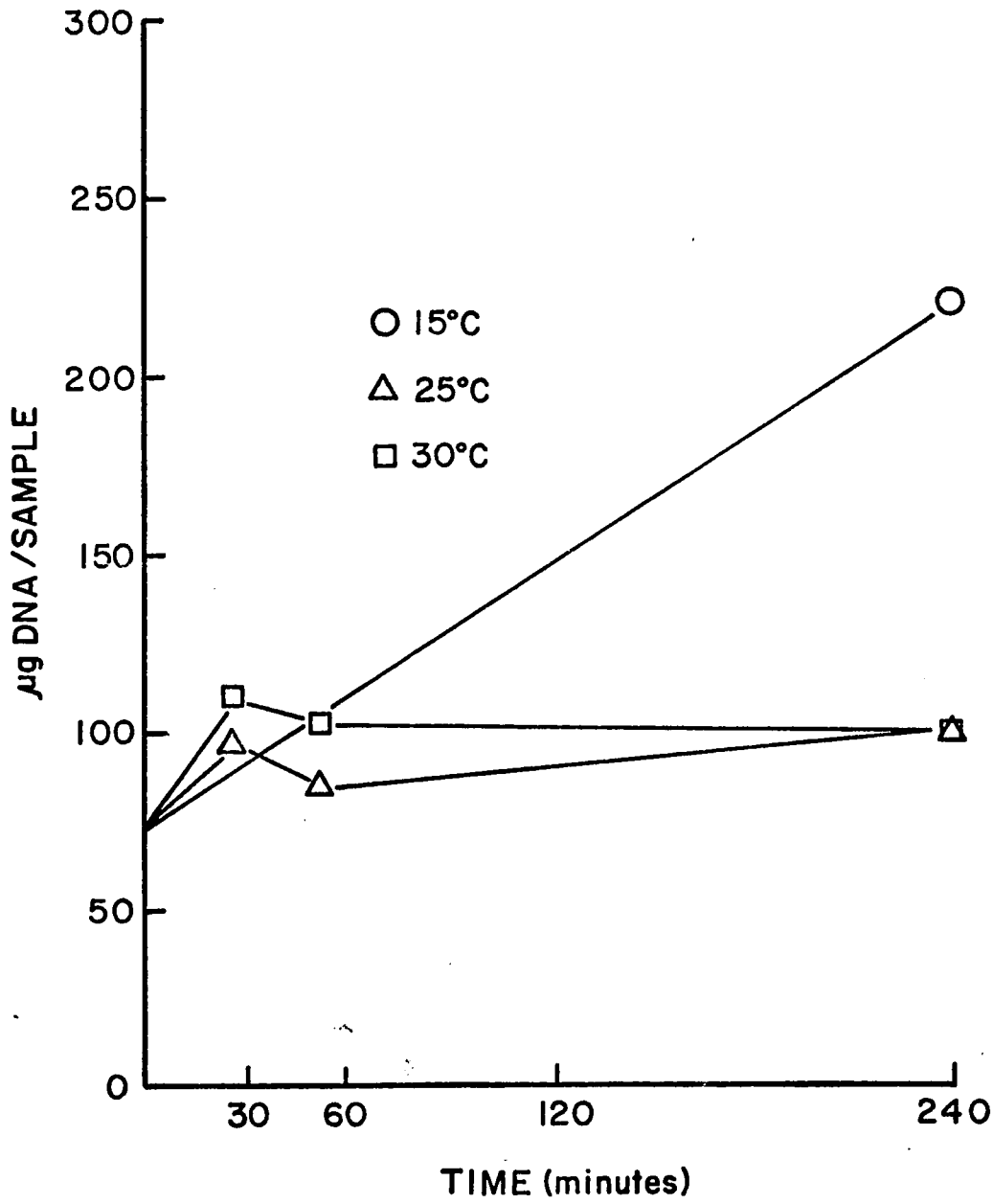

Figure 11. The effect of permissive and restrictive growth temperatures on the DNA content of L. stokesii.

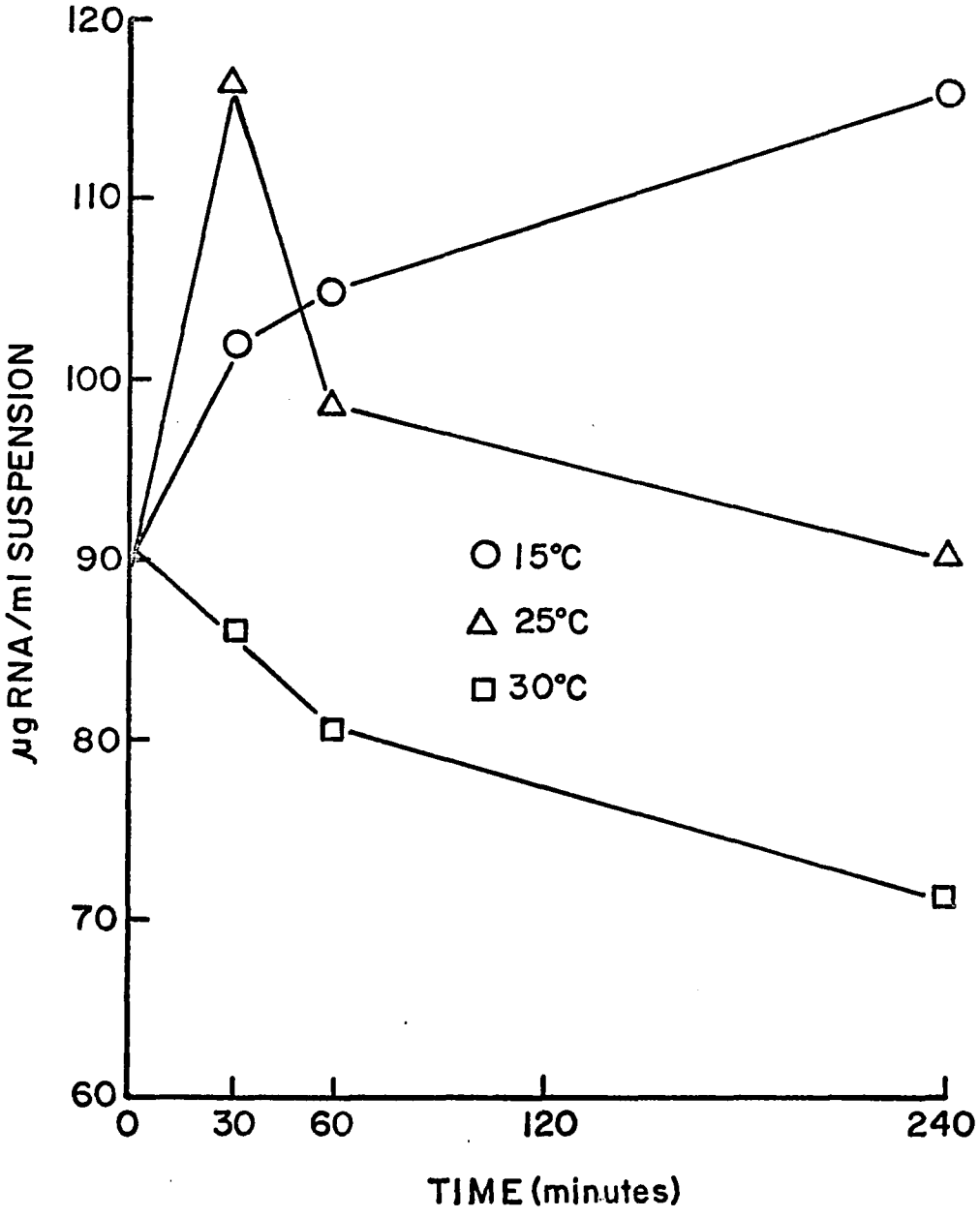

Figure 12. The effect of permissive and restrictive growth temperatures on the RNA content of cells of L. stokesii.

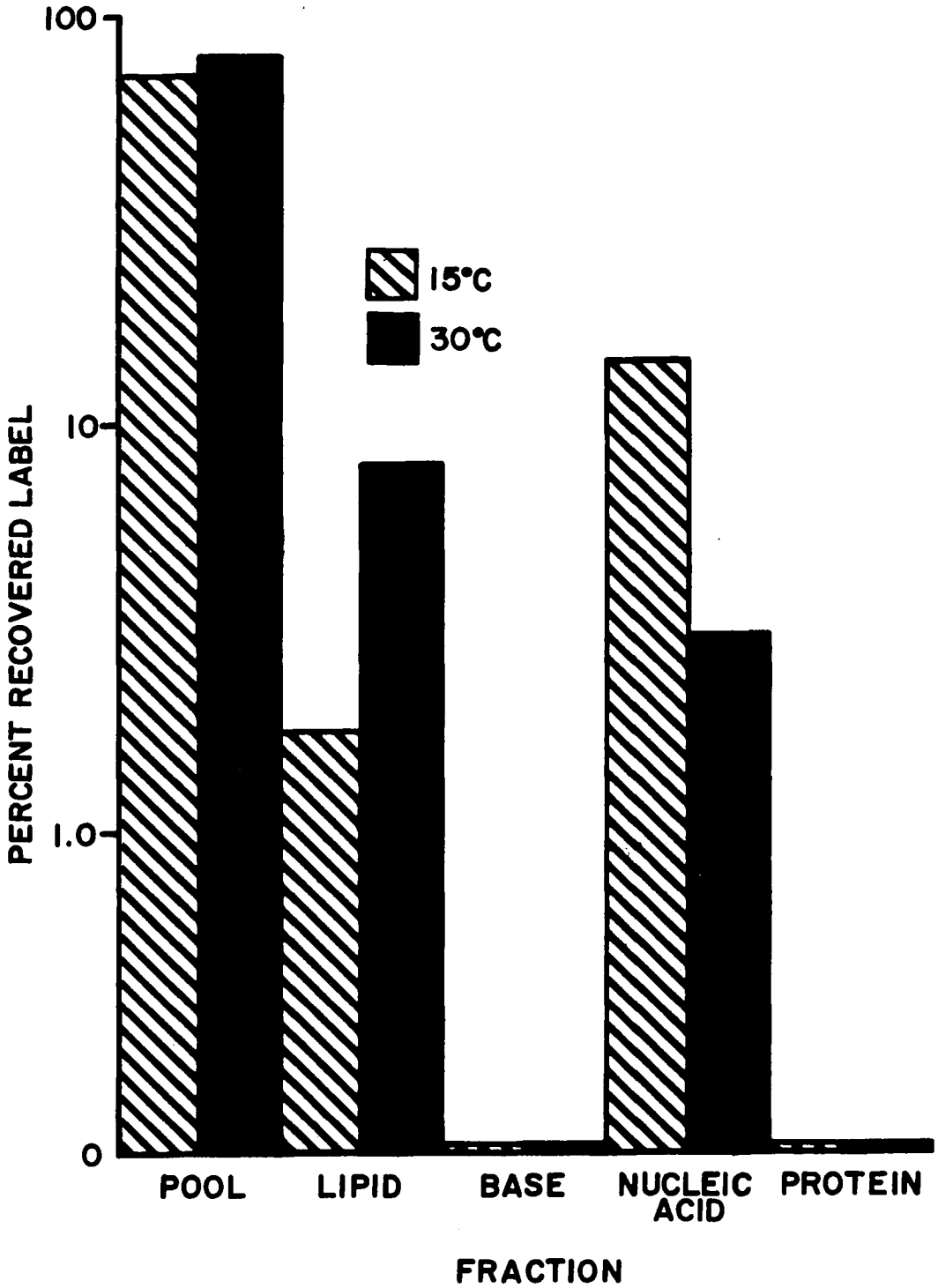

Figure 13. The effect of permissive and restrictive growth temperatures on incorporation of radioactive thymine into cellular fractions of L. stokesii.

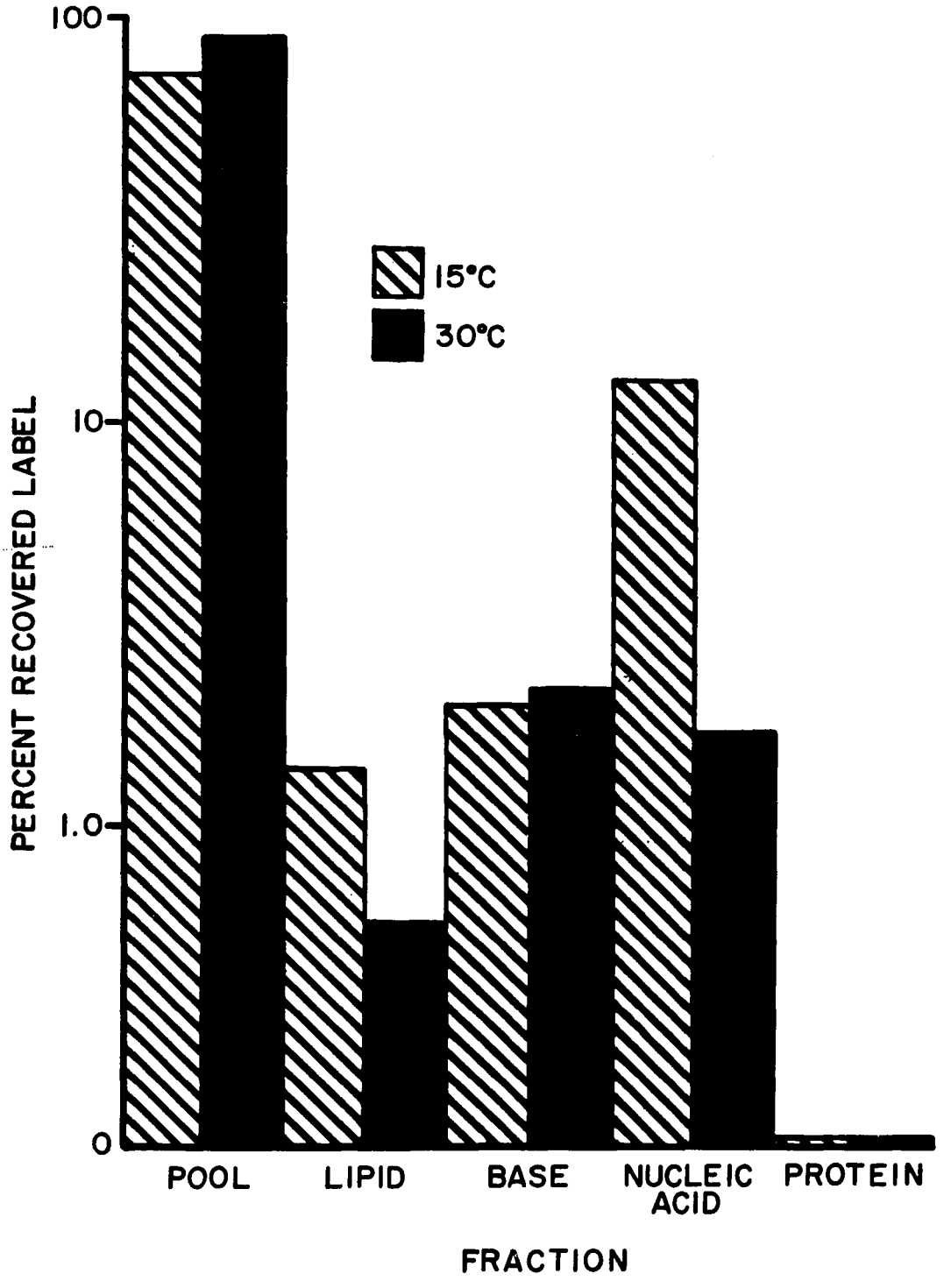

Figure 14. The effect of permissive and restrictive growth temperatures on the incorporation of radioactive uracil into cellular fractions of L. stokesii.

amounts of label found in the pool fraction were approximately the same in cultures incubated at both temperatures, although slightly less label was found in the 15°C pool fraction. No label was recovered from the base fraction or the protein fraction at either incubation temperature.

The incorporation of radioactive uracil into the various cell fractions is shown in Figure 14. Over five times as much label was recovered from the 15°C nucleic acid fraction as from the 30°C nucleic acid fraction. Approximately 10% less label was found in the pool fraction at 15°C than at 30°C. No uracil label was recovered from the protein fraction.

The Effect of Permissive and Restrictive Growth Temperatures on the Levels of ATP in Cells of L. stokesii

The amount of ATP available is intimately involved with the synthetic capacity of the cell. For this reason the levels of ATP in L. stokesii during heat stress were determined. Cells were cultured in J-J medium at 15°C to 100 Kletts, suspended in growth medium, and incubated at 15°C, 25°C, and 30°C. Samples were removed at intervals and assayed for ATP by the method of Patterson et al. (1970). The results are shown in Figure 15. At 15°C the level of ATP in the cell remains relatively constant over the 4-hr incubation period. At 25°C and 30°C the levels of ATP decrease rapidly for the first 2 hr of the heat treatment and then increase over the remaining 2-hr period. Increases in ATP levels in dead or moribund cells have been reported by Malcolm

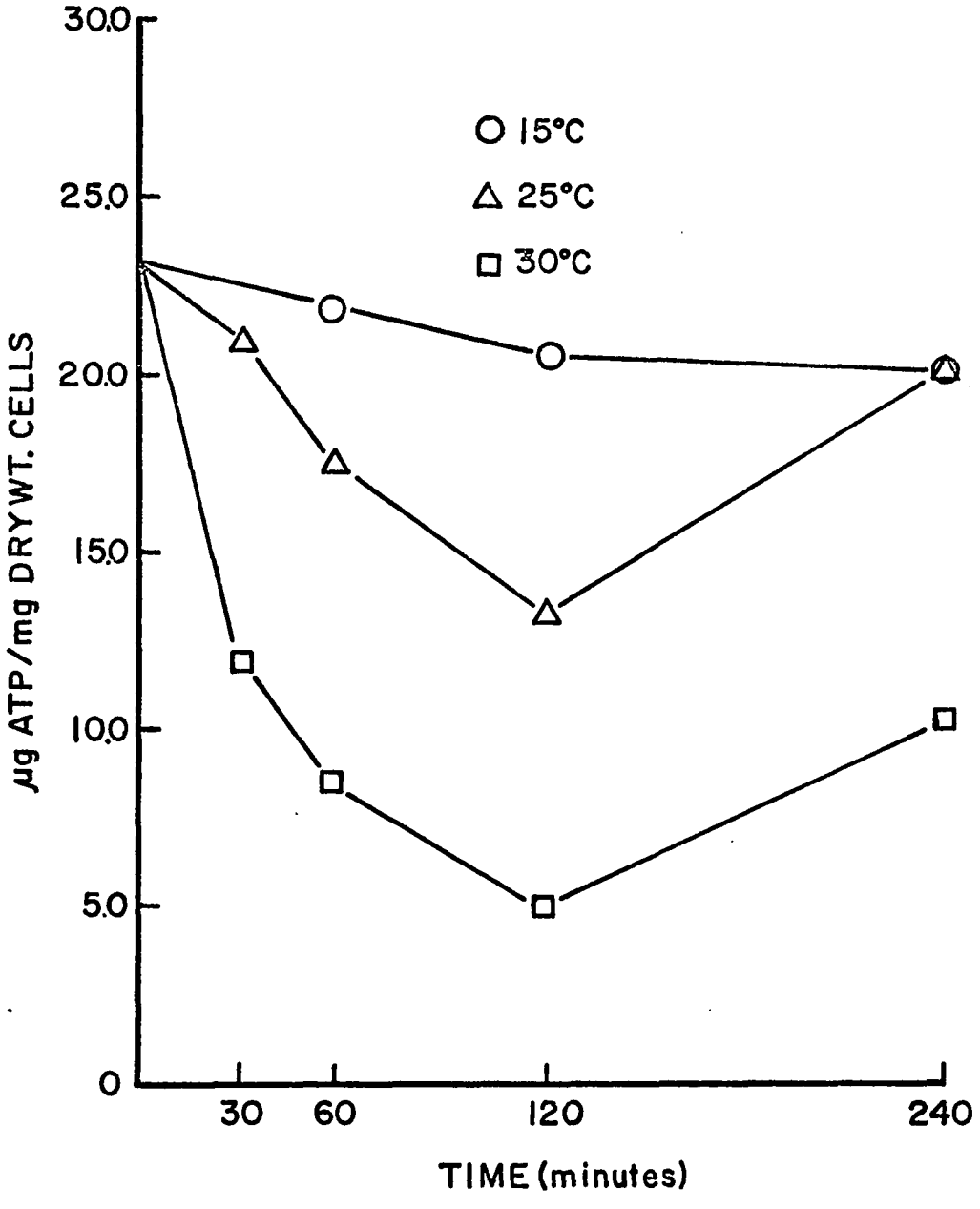

Figure 15. The effect of permissive and restrictive growth temperatures on the levels of ATP in the cells of L. stokesii.

(1968) in Micrococcus cryophilus. These data indicate that the apparatus for the supply of ATP is still functional after 4 hr of incubation at either 25°C or 30°C.

DISCUSSION

The investigation of thermal death in microorganisms has been an active area of research in recent years. The only agreement about the mechanisms of thermal death is that it happens at relatively low temperatures in psychrophiles. Nearly every system in the cell has been pointed to as a cause of thermal death including enzyme activity and synthesis, cell membrane failure, lack of synthetic activity, and failure of energy metabolism. Studies with psychrophilic organisms have been centered on protein synthesis and in the loss of activity of enzymes involved in ATP production. Unfortunately, few of these investigations were conducted at temperatures near the maximum growth temperature of the organism involved. Evison and Rose (1965) transferred a psychrophilic cryptococcus to temperatures three to five degrees above the maximum growth temperature and they observed loss in activity of several TCA cycle enzymes. Nash and coworkers (1968, 1969) have reported on other psychrophilic yeasts and have proposed theories about thermal death in these organisms. This dissertation considers the responses of three obligately psychrophilic yeasts to temperatures close to but above the maximum growth temperature. Even in the crudest measurements of the response of these organisms to heat there are differences in their responses to heat stress. The viability of both L. frigidum and Candida species P-25 decreases immediately upon exposure to 25°C, a temperature only five degrees above the maximum growth temperature of these organisms. This was not observed in the case of

L. stokesii. Viability did not decrease until after 2 hr of incubation at 25°C. Further differences were noted when the respiratory activity of the three yeasts was compared. At 30°C after 2 hr of incubation, L. frigidum (P-8) and Candida sp. P-25 showed no measurable respiratory activity although they retained 32% and 26% of their viability, respectively. Leucosporidium stokesii retained almost half of its respiratory activity even though 87% of the cells in the culture were nonviable. These data suggest that respiratory failure may be involved in the determination of the maximum growth temperature of L. frigidum and Candida sp. P-25 but that a different mechanism is involved with L. stokesii.

Robinson and Morita (1966) reported that a psychrophilic bacterium leaked ultraviolet absorbing material which correlated with its loss in viability. Hagen, Kushner, and Gibbons (1964) established that death preceded lysis and leakage in a psychrophilic bacterium. Malcolm (1968) contended that temperature-induced membrane damage and leakage are not causes of death in the psychrophilic bacterium M. cryophilus. Investigations on leakage of material from the yeast P-7 (C. nivalis) indicated leakage of ultraviolet-absorbing material from the cell during heat injury (Nash and Sinclair 1968).

In the studies reported here leakage was correlated with viability in the three yeasts under study and again a differing pattern emerged. Of the three yeasts investigated only L. frigidum leaked 260 nM absorbing material at a temperature of 25°C within 2 hr. Candida sp. P-25 showed the least amount of leakage at 25°C, but at 30°C

released more ultraviolet-absorbing material than any other yeasts tested. Leucosporidium stokesii did not leak at 25°C in the first 2 hr and displayed the least amount of leakage at 30°C of any of the yeasts tested. Even with these differences it is possible to compare leakage and thermal death. Leakage at 25°C is not evident in L. stokesii and Candida sp. P-25 until after 10% of the population is dead and does not exceed leakage from the 15°C controls until in the case of L. stokesii the population has been reduced by over 40%. Candida sp. P-25 does not leak OD_{260} absorbing material to a greater degree than the control (15°C) cultures at any time during treatment at 25°C. Appreciable leakage in all three yeasts does not take place at 30°C until the majority of the cells in the population are dead. These data agree with those of Hagen et al. (1964) in that death of heat-stressed cells preceded lysis.

The above data as well as the data on dye permeability and reversibility of membrane damage argue that heat-induced failure of the cell membrane is not the primary cause of death at least with respect to L. stokesii. In this organism the entry of neutral red into the cytoplasm of cells incubated at 30°C is not appreciably greater than the 15°C controls until 97.5% of the population is dead. At 25°C the ability of the cell to take up leucine is impaired but not stopped. Impairment of function is almost immediately reversible when the cells are returned to 15°C incubation.

Investigations of the source of material leaking from heat-stressed cells have not been made, although Malcolm (1968) suggested

that the source might be degradation of intracellular RNA. Loss of RNA from heat-stressed cells has been noted by Malcolm (1968) in M. cryophilus and was also detected by Harder and Veldkamp (1968) in a psychrophilic pseudomonad. Loss of RNA was also detected from L. stokesii in this investigation. The possibility of enzymatic degradation of RNA by the cell in response to the heat treatment was tested in L. stokesii by two methods including radioactive tracer leakage from prelabeled cells and assay for heat-induced RNAse activity. Cell-free extracts were prepared from cells of L. stokesii by two different methods and the assay of RNAse was undertaken at various times during the course of heat stress, and in no case was RNAse activity detected. The radiotracer experiments showed that the leakage products came from the pool fraction of the cell and not the nucleic acid fraction. Nash and Grant (1969) have shown reduced activity of ribosomes following exposure of L. stokesii to heat. Physical degradation of the ribosomes, however, occurred only at a temperature of 45°C, a temperature much higher than those employed in this study. Nash and Grant (1969) also reported that L. stokesii (P-16) RNA contained less uracil than did RNA from the mesophile (C. utilis) used for comparison. The earlier data of Nash and Sinclair (1968) on the related yeast C. nivalis (P-7) indicated that 5'-uracil monophosphate leaked from the cells during heat stress. Since the uracil content of L. stokesii RNA is low, the pools might be expected to contain elevated levels of uracil. The data on leakage of L. stokesii fit well into this picture as it was shown that leakage occurred primarily from the pool fraction of the cell.

The data on protein synthesis in psychrophiles suggest that protein synthesis is heat-sensitive in many cases. Malcolm (1968) defined the initial heat-induced lesion in M. cryophilus as the sensitivity of amino acid activating systems and their cognate t-RNAs. Nash et al. (1969) demonstrated that L. stokesii contained amino acyl-t-RNA synthetases sensitive to 35°C heat for 30 min. In this study, temperatures closer to the maximum growth temperature were employed and a somewhat different situation was observed. At 25°C no impairment of the protein synthetic apparatus was noted, as indicated by both chemical and radioactive tracer data. At 30°C, however, incorporation of arginine took place for the first 30 min of treatment even though viability of the population decreased. These data suggest that at temperatures close to the maximum growth temperature, 25°C, protein synthesis per se is not inactivated. Therefore, impairment of protein synthesis is not a primary cause of death in L. stokesii.

Nash and Grant (1969) noted that ribosomes were rapidly affected by heat treatment and lost their capacity to bind charged t-RNA after exposure to temperatures from 10°C to 25°C above the maximum growth temperature of 20°C in L. stokesii. This may result in the loss of protein synthesis or the cell may synthesize increased amounts of RNA. This situation could be analogous to the compensatory RNA synthesis reported by Harder and Veldkamp (1968). My data indicate that at 25°C there is an apparent stimulation of RNA production during the initial stages of heat injury and a subsequent decrease in the amount of RNA in the culture over the remaining period of incubation. The above

response of RNA synthesis to heat stress correlates well with the loss of viability of L. stokesii at 25°C. At 30°C the amount of RNA in the culture decreased immediately. Loss of viability was also immediate at 30°C.

Synthesis of both RNA and DNA are initially stimulated by exposure to supermaximal temperatures. Whole cell uptake of radioactive uracil and thymine as well as chemical determinations of nucleic acid content showed initial stimulation. To a limited extent these data correlate with data collected on the levels of ATP found in the cells of L. stokesii during heat stress. As the uptake and incorporation of uracil and thymine are stimulated and the production of RNA and DNA increased at supermaximal temperatures, the level of ATP in the cells decreased rapidly. After uptake stopped and RNA and DNA production ceased, the levels of ATP in the cells increased. Moreover, at 25°C the ATP concentration in the cells increased during the 4-hr incubation period to the level initially present. This phenomenon has also been reported by Malcolm (1968) in the psychrophilic bacterium, M. cryophilus.

In conclusion, it has been shown that at temperatures close to the maximum growth temperature of the obligately psychrophilic yeast Leucosporidium stokesii, the lesion responsible for loss in viability does not appear to be related to inactivation of protein synthesis, failure of the cell membrane, lack of ATP or its production, nor enzyme-induced degradation of intracellular RNA. Of all the parameters examined, the only data congruent with the loss in viability of L. stokesii

at supermaximal, restrictive growth temperatures are the decrease of the RNA content of the cells.

A possible sequence of events in the heat injury of L. stokesii and subsequent death of this organism might be: (1) temperature stimulation of cellular processes including protein synthesis, DNA synthesis and RNA synthesis, (2) increased utilization of ATP as a consequence of increased synthetic activity, (3) loss of ability of ribosomes to direct polypeptide formation, (4) loss of the ability of the cell to produce RNA and the subsequent loss of RNA from the cell, and (5) loss of RNA synthetic capability and consequent loss of viability.

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