A Comparative Study on the Biochemical Bases of the Maximum Temperatures for Growth of Three Psychrophilic Micro-Organisms

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SUMMARY

Three psychrophilic micro-organisms (strains of *Arthrobacter*, *Candida* and *Corynebacterium erythrogenes*) were capable of growth for a period when exponential-phase cultures in chemically defined media were transferred from temperatures at or near the optima for growth (20, 10 and 15°, respectively), to 37, 25 and 30°, respectively. The latter temperatures were 3–5° above the maxima for the growth of the organisms in freshly inoculated cultures. Growth at the higher temperatures was greatest with the *Candida* and least with the *Arthrobacter*. Cultures of the *Arthrobacter* and *Candida* grew when transferred back to the optimum temperatures for growth, after a lag which increased with the length of time that the cultures had spent at the higher temperatures. *C. erythrogenes* cultures grew almost immediately after they were transferred back to the optimum growth temperature. Growth of the organisms at the higher temperatures was not affected by supplementing cultures with bacteriological peptone and/or yeast extract. There was a rapid decline in the viability and in the rates of respiration of endogenous reserves and of exogenous glucose and pyruvate when *Arthrobacter* and *Candida* cultures were transferred to the higher temperatures. But with *C. erythrogenes* the respiratory activities were not so markedly affected by the change in incubation temperature, while the viability of this bacterium increased slightly after the transfer of cultures to the higher temperature. The activities of many of the tricarboxylic acid cycle enzymes in *Arthrobacter* and *Candida* were diminished after the transfer of organisms from the optimum temperature to one above the maximum for growth; but the activities of these enzymes in *C. erythrogenes* were less affected by the change in incubation temperature. There was no marked intracellular accumulation or excretion of ultra-violet-absorbing compounds by the organisms after the transfer of cultures to the higher temperatures. The results are discussed in relation to the biochemical factors which may determine the maximum temperatures for growth of these organisms.

INTRODUCTION

Psychrophilic micro-organisms differ from mesophils in having lower minimum temperatures for growth. But the maximum temperatures for growth of psychrophils can vary from around 18°, as with a strain of *Serratia marcescens* (Kates & Hagen, 1964) to between 40 and 50° which is in the range of maximum temperatures for growth of many mesophils. Very little is known about the biochemical bases of the maximum temperatures for growth of micro-organisms, although several factors are thought to be involved including the denaturation of enzymes (Chick,

1910; Edwards & Rettger, 1937), denaturation and possible degradation of DNA (Marmur & Doty, 1959) and RNA (Califano, 1952; Strange & Shon, 1964) and changes in the properties of membrane lipids (Luzzati & Husson, 1962; Byrne & Chapman, 1964; Hagen, Kushner & Gibbons, 1964). Enzyme denaturation is usually assumed to be a major factor, and excellent agreement between the maximum temperatures for growth of several bacteria and the minimum temperatures at which certain of their respiratory enzymes were inactivated was reported by Edwards & Rettger (1937). By using a technique in which exponentially growing organisms were transferred from the optimum temperature for growth to a temperature some 3–5° above the maximum for growth, Hagen & Rose (1962) showed that the low maximum temperature for growth of a psychrophilic *Cryptococcus* (about 28°) was determined at least in part by the heat-sensitive nature of one or more of the tricarboxylic acid (TCA) cycle enzymes. Upadhyay & Stokes (1963) reported the presence of a heat-sensitive formate hydrogenlyase in a psychrophilic bacterium and Burton & Morita (1963) showed that the malate dehydrogenase in a psychrophilic marine bacterium was also abnormally sensitive to heat denaturation, although in neither of these reports was there evidence that the heat sensitivity of the enzyme determined the maximum temperature for growth of the given organism. The present paper records the results of a comparative study on the biochemical bases of the maximum temperatures for growth in three psychrophilic micro-organisms which have maximum temperatures within the range 22–33°.

METHODS

Organisms. The origin and maintenance of the strains of *Arthrobacter* (No. R22/3A), *Candida* (No. A3E-2) and *Corynebacterium erythrogenes* (NCMB 5) examined here were described by Rose & Evison (1965).

Experimental cultures. The strains of *Arthrobacter* and *Corynebacterium erythrogenes* were grown in the defined medium (pH 6.7) described by Rose & Evison (1965) and the strain of *Candida* in the glucose + salts + vitamins medium (pH 4.5) of Rose & Nickerson (1956) supplemented with D-biotin (2.0 µg./l.). Portions (100 ml.) of bacterial or yeast medium in 350 ml. conical flasks were prepared as described by Rose & Evison (1965). In certain experiments, cultures (6 ml.) were grown in Samco tubes covered with anodized aluminium caps (Oxo Ltd., Queen Street Place, London, E.C. 4; Northam & Norris, 1951). Solutions of substances were occasionally added to these 6 ml. cultures as described later. These solutions were adjusted to pH 4.5 when added to yeast cultures, or to pH 6.7 when added to bacterial cultures, and were sterilized separately by autoclaving momentarily at 115°. Portions of sterile medium were inoculated as described by Rose & Evison (1965). Cultures were incubated statically at the temperatures stated. Growth was measured turbidimetrically in Samco tubes with the Hilger 'Spekker' absorptionmeter (Model H760) with neutral green-grey H 508 filters and a water blank. Turbidity readings were related to dry weight by using a calibration curve for each organism.

Viable counts of organisms in cultures were made by spreading samples (0.1 ml.) from successive ten-fold dilutions in water on well-dried plates of malt wort-agar (10%, w/v, spray-dried malt extract, 'Muntona', Munton and Fison, Ltd., Stow-

market, Suffolk, +2 %, w/v, agar, +0.5 %, w/v, NaCl) for the yeast, or on plates of nutrient agar for the bacteria. Triplicate plates were used with each dilution. Plate cultures of the *Candida* were incubated at 10° for 144 hr, those of the *Arthrobacter* at 20° for 48 hr and of *C. erythrogenes* at 20° for 72 hr. The colonies on plates which had received suitably diluted portions were counted with an electric colony counter (Sintacell, Ltd., London, E.C. 4). The contents of viable organisms in cultures are expressed as the number/mg. dry wt. organism.

Respirometry. The respiratory activities of organisms were determined as described by Umbreit, Burris & Stauffer (1964) with the constant volume Warburg respirometer (B. Braun, Melsungen, West Germany; Model S 85) fitted with cooling coils through which was circulated cold water from a low temperature water bath (model LB 405; Grant Instruments Ltd., Barrington, Cambridge) when required. Organisms were harvested from cultures at the times indicated in a refrigerated centrifuge at 0°. The yeast was washed three times with phosphate buffer (M/15 KH_2PO_4 ; pH 4.5) and the bacteria with 0.85 % (w/v) NaCl. The washed organisms were suspended in a volume of phosphate buffer (pH 4.5 for the yeast; pH 7.0, Gomori, 1955, for bacteria) to a concentration suitable for use in the Warburg respirometer. The centre well of each Warburg flask contained 0.2 ml. 10 % (w/v) KOH and a small filter-paper wick. A portion of suspension containing a suitable quantity of organisms was added to the flask and the total volume adjusted to 2.5 ml. with buffer. The side arm contained 0.3 ml. of a solution (2.5 %, w/v; pH 4.5 or 7.0) of oxidizable substrate, or 0.3 ml. water when measuring the respiration of endogenous reserves. After the Warburg flasks had been attached to the manometers, they were equilibrated in the water bath for 10–30 min., depending upon the temperature of the bath. After the manometer taps had been closed and the substrate tipped from the side arm, the uptake of oxygen by the organisms was followed over a period of 1 hr. The respiratory activities are quoted as the Q_{O_2} values ($\mu\text{l.}$ oxygen consumed/mg. dry wt. organism/hr) for the respiration of endogenous reserves, and of exogenous substrate after subtracting the value for the respiration of endogenous reserves.

Preparation of cell extracts. Extracts of organisms for use in the measurement of enzyme activities were prepared by ballistic disintegration. Organisms were harvested from cultures by centrifugation at 0° in a refrigerated centrifuge. Bacteria were washed twice with 0.85 % (w/v) NaCl and the yeast with phosphate buffer (pH 4.5). The equivalent of 50–200 mg. dry wt. organisms was washed once with ice-cold water, suspended in 5.0 ml. ice-cold water and shaken with 3 g. Ballotini beads (Grade 12) in a Mickle tissue disintegrator (Mickle, 1948) as described by Ahmad & Rose (1962). Cell-free extracts were obtained by centrifuging the suspension of disrupted organisms at 1300g for 20 min. at 0°. Extracts were either used immediately or stored at –20° until required. The protein contents of the extracts were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine plasma albumin (L. Light and Co., Ltd., Colnbrook, Bucks.) as a standard. Acid-soluble ultraviolet (u.v.)-absorbing compounds were extracted from portions of washed organisms (containing equiv. 5 mg. dry wt. organism) by using 5 % (w/v) trichloroacetic acid as described by Ahmad, Rose & Garg (1961).

Enzyme assays. The enzyme nomenclature used is that recommended in the Report

of the Commission on Enzymes of the International Union of Biochemistry, 1961, although only the suggested trivial names are used for the dehydrogenases studied since the experimental results do not permit precise identification of the enzymes concerned. The activities of all of the enzymes studied were calculated from initial reaction velocities determined over a period during which plots of the amount of substrate changed against time were linear. All activities are expressed as specific activities (μ mole-substrate consumed/mg. extract protein/hr).

The activities of aconitate hydratase, isocitrate dehydrogenase, fumarate hydratase and L-malate dehydrogenase were measured spectrophotometrically by using the S.P. 500 quartz spectrophotometer fitted with a constant temperature cuvette housing (Unicam Ltd., Cambridge) through which was circulated water at an appropriate temperature. Reactions were carried out in 1 cm. quartz cuvettes and all reaction constituents except cell extracts, which were kept at 0°, were equilibrated in the cuvettes at the test temperature before starting the reaction. The temperature of the reaction mixture in the cuvettes was measured by using either a mercury thermometer or a Rustrak miniature temperature recorder coupled to a hypodermic thermistor which fitted down the inside of the cuvette (Grant Instruments Ltd., Toft, Cambridge).

Aconitate hydratase (citrate (isocitrate) hydrolyase; EC 4.2.1.3) was assayed by a method based on that of Racker (1950) which depends on measuring the increase in extinction at 240 m μ attendant upon the conversion of citrate to *cis*-aconitate. Each cuvette was charged with sodium citrate (87 μ moles; pH 7.4) and sodium phosphate buffer (145 μ moles; pH 7.4) in 2.9 ml. water. The reaction was started by adding 0.1 ml. of a suitably diluted portion of cell extract containing approximately 100 μ g. protein; 0.1 ml. water was added to the control cuvette. The increase in extinction at 240 m μ , caused by the formation of *cis*-aconitate, was followed at 30 sec. intervals for a period of 5 min. Specific activities were calculated using the value for the extinction in 3.0 ml. water of 1 μ mole *cis*-aconitate at 240 m μ quoted by Williams & Rainbow, (1964).

Isocitrate dehydrogenase (NADP-linked) was assayed by following at 340 m μ the increase in extinction on reduction of NADP (Ochoa, 1948; Kornberg & Pricer, 1951). Each cuvette contained sodium DL-isocitrate (0.5 μ mole; pH 7.0), potassium phosphate buffer (100 μ moles; pH 7.0), NADP (0.5 μ mole), MgCl₂ (10 μ moles) in 2.9 ml. water. The reaction was started by adding 0.1 ml. of diluted cell extract containing about 100 μ g. protein, and the increase in extinction at 340 m μ was followed at 30 sec. intervals over a period of 3 min., with a blank reaction mixture lacking cell extract. The extinction of a second blank reaction mixture containing all of the constituents except DL-isocitrate was measured at the beginning and at the end of the period of observation. Specific activities were calculated from the change in extinction using the molar extinction coefficient for NADPH₂ quoted by Horecker & Kornberg (1948).

Fumarate hydratase (L-malate hydro-lyase; EC 4.2.1.2) activity was assayed using a method based on that of Racker (1950) which depends on measuring the decrease in the extinction at 300 m μ attendant on the conversion of fumarate to L-malate. Each cuvette contained sodium fumarate (49 μ moles; pH 7.3) and sodium phosphate buffer (95 μ moles; pH 7.3) in 2.9 ml. water. The reaction was started by adding 0.1 ml. of cell extract containing about 400 μ g. protein; 0.1 ml. water was

added to the control reaction mixture. The decrease in extinction at 300 $m\mu$, caused by the decrease in the concentration of fumarate, was followed at 30 sec. intervals over a period of 5 min. Specific activities were calculated using a value for the extinction of 1 μ mole fumarate in 3.0 ml. at 300 $m\mu$ of 0.015.

Two methods were used for assaying L-malate dehydrogenase (NAD-linked). The activity of this enzyme in cell extracts of the *Candida* was assayed by a modification of the method of Beaufay, Bendall, Baudhuin & de Duve (1959) with L-malate as substrate and following at 340 $m\mu$ the increase in the extinction of the reaction mixture on the reduction of NAD. Each cuvette contained tris buffer (58 μ moles; pH 8.5), potassium-L-malate (600 μ moles; pH 8.5), NAD (0.7 μ mole; pH 8.5), NaCN (30 μ moles; pH 8.5) and ethylenediaminetetra-acetic acid (EDTA) (3 μ moles; pH 8.5) in 2.9 ml. water. The constituents were incubated at room temperature (18–22°) for 2 hr to allow the NAD and NaCN to equilibrate. The reaction was started by adding 0.1 ml. of diluted cell extract containing about 100 μ g. protein and the increase in extinction at 340 $m\mu$ was followed at 30 sec. intervals for a period of 4 min. Two blank reaction mixtures were used with each experiment, one lacking NAD and cell extract and the other lacking L-malate. Specific activities of the *Candida* extracts were calculated by using the molar extinction coefficient for NADH₂ reported by Horecker & Kornberg (1948). Only very slight malate dehydrogenase activity was detected in extracts of the *Arthrobacter* or of *Corynebacterium erythrogenes* by this assay method. Malate dehydrogenase activity could, however, be assayed in extracts of these bacteria using oxaloacetate as substrate and following the decrease in extinction caused by the oxidation of NADH₂ (Ochoa, 1955a). Each cuvette contained sodium phosphate buffer (60 μ moles; pH 7.4), potassium oxaloacetate (0.76 μ mole; pH 7.4) and NADH₂ (0.15 μ mole; pH 7.4) in 2.9 ml. water. The reaction was started by adding 0.1 ml. of a suitably diluted portion of cell extract containing about 100 μ g. protein and the decrease in extinction at 340 $m\mu$ was followed at 30 sec. intervals over a period of 3 min. Specific activities were calculated by using the molar extinction coefficient for NADH₂ quoted by Horecker & Kornberg (1948). Extracts of the *Candida* did not show malate dehydrogenase activity when assayed using this method even when the pH value of the reaction mixture was raised to 8.4 or lowered to 5.8.

The activities of pyruvate, 2-oxoglutarate and succinate dehydrogenases in cell extracts were assayed manometrically by the conventional constant volume respirometer technique (Umbreit *et al.* 1964). Pyruvate dehydrogenase activity was assayed by measuring carbon dioxide evolution with lithium pyruvate as substrate and potassium ferricyanide as electron acceptor (Jaganathan & Schweet, 1952). Each Warburg flask was charged with lithium pyruvate (100 μ moles), sodium bicarbonate (50 μ moles), MgCl₂ (20 μ moles) and thiamine pyrophosphate (200 μ g.; adjusted to pH 6.0 just before use). These constituents were added as a solution which had been flushed with carbon dioxide gas for 2–3 min. A portion of cell extract containing 3–8 mg. protein and water to a volume of 3 ml. were also added to the flask, the side arm of which contained potassium ferricyanide (100 μ moles). The flasks were attached to the manometers, and flushed with carbon dioxide gas for 10 min., with a glass manifold to ensure even gassing. The manometer units were then quickly transferred to the water bath and equilibrated for 10 min. After the potassium ferricyanide had been tipped in from the side arm, the evolution of

carbon dioxide was followed during a period of 10 min. Control flasks contained all of the constituents of the reaction mixture except cell extract. Specific activities were calculated from the amount of carbon dioxide evolved.

2-Oxoglutarate dehydrogenase activity in cell extracts was assayed by measuring carbon dioxide evolution in a system which contained 2-oxoglutarate as substrate and potassium ferricyanide as electron acceptor (Sanadi, Littlefield & Bock, 1952). The main compartment of each Warburg flask was charged with sodium 2-oxoglutarate (50 μ moles), sodium bicarbonate (400 μ moles), thiamine pyrophosphate (200 μ g.; adjusted to pH 6.9 just before use) and MgCl_2 (20 μ moles). These constituents were added as a solution that had been flushed for 2–3 min. with carbon dioxide gas. The solution in the Warburg flasks was supplemented with bovine plasma albumin (L. Light and Co. Ltd., Colnbrook, Bucks; 30 mg.), a portion of cell extract containing 3–8 mg. protein and water to a volume of 3 ml. The side arm of the flask contained potassium ferricyanide (50 μ moles). The flasks were attached to the manometers, flushed with carbon dioxide gas for 10 min., quickly transferred to the Warburg bath and equilibrated for 10 min. After the potassium ferricyanide had been added from the side arm, the evolution of carbon dioxide was recorded during 10 min. Control flasks contained all of the constituents of the reaction mixture except cell extract. Specific activities were calculated from the amount of carbon dioxide evolved.

Succinate dehydrogenase activity in cell extracts was assayed by measuring the amount of oxygen uptake with succinate as a substrate in the presence of phenazine methosulphate as electron carrier (Bernath & Singer, 1962). Each Warburg flask was charged with sodium phosphate buffer (150 μ moles; pH 7.6), KCN (30 μ moles; pH 7.6), cell extract containing 3–4 mg. protein and water to 3.0 ml. Sodium succinate (60 μ moles; pH 7.6) and phenazine methosulphate (0.2, 0.1, 0.07, 0.05 or 0.04 ml. of a 1%, w/v, solution) were added to the side arm. KCN was added last, the flasks immediately attached to the manometers and the stopcocks closed. The excess pressure was released momentarily after the units had been placed in the Warburg bath. After equilibrating for 10 min. the contents of the side arms were tipped into the flasks and the uptake of oxygen was recorded during 20 min. Control flasks contained all of the constituents of the reaction mixture except cell extract. The reciprocal of the Q_{O_2} value (calculated over a period of 2–12 min.) was then plotted against the reciprocal of the concentration of phenazine methosulphate and the oxygen uptake extrapolated to infinite phenazine methosulphate concentration. This value for the oxygen uptake was used to calculate the specific activities of succinate dehydrogenase in the extracts (Bernath & Singer, 1962).

The method of Ramakrishnan & Martin (1954) was used for assaying the citrate synthase (citrate oxaloacetate-lyase (Co A-acetylating); EC 4.1.3.7) activities of cell extracts. This involved using acetyl phosphate, coenzyme A and transacetylase to generate acetyl coenzyme A which was then allowed to react with oxaloacetate to give citrate in a reaction catalysed by citrate synthase. In this system, the amount of acetyl phosphate used is proportional to the amount of citrate synthase present (Ochoa, 1955*b*). The reaction was carried out in Warburg flasks which were placed in a tray of ice and charged with potassium phosphate buffer (25 μ moles; pH 7.4), potassium oxaloacetate (20 μ moles; pH 7.4), acetyl phosphate (10 μ moles) coenzyme A (0.05 μ mole), L-cysteine (10 μ moles; pH 7.4), MgCl_2 (4 μ moles), trans-

acetylase preparation (0.04 ml. containing approximately 0.8 mg. protein; Ramakrishnan & Martin, 1954), a portion of cell extract containing 0.5–4.0 mg. protein and water to 1 ml. The transacetylase preparation was obtained from *Escherichia coli* strain NRC 482 as described by Ramakrishnan & Martin (1954), except that the extract was not fractionated with ammonium sulphate. Control flasks lacking cell extract were set up. The Warburg flasks were placed on the manometers and immediately incubated at the test temperature for 20 min. The flasks were then removed from the manometers and placed in the tray of ice for 5 min., and the acetyl phosphate remaining in the reaction mixture was determined by the hydroxamate method of Lipmann & Tuttle (1945). Specific activities were calculated from the amount of acetyl phosphate used.

RESULTS

Effect of change of incubation temperature on growth

Exponential-phase cultures of a psychrophilic strain of *Cryptococcus* were shown by Hagen & Rose (1961) to grow rapidly for a period, at a temperature about 3° above the maximum for growth in freshly inoculated culture, when they were previously incubated at or near the optimum temperature for growth (16°).

Fig. 1. Growth of cultures (6 ml.) of *Arthrobacter* (a), *Candida* (b), *Corynebacterium erythrogenes* (c) after transfer from a temperature at or near the optimum for growth (●—●) to one 3–5° above the maximum for growth (○—○). Cultures of *Arthrobacter* were transferred to 37° after 62, 135 and 255 hr incubation at 20°; cultures of *Candida* to 25° after 90, 114 and 191 hr incubation at 10°; *C. erythrogenes* cultures to 30° after 96, 192 and 264 hr at 15°.

The effects of transferring cultures of each of the organisms used in the present work from a temperature at or near the optimum for growth to one 3–5° above the maximum for growth in freshly inoculated cultures are shown in Fig. 1. All three organisms grew to some extent after transfer to the higher temperatures. The amount of growth at the higher temperatures was greatest with the *Candida* and least with the *Arthrobacter*, but none of the organisms grew to the same extent as did the psychrophilic *Cryptococcus* (Hagen & Rose, 1961) after transfer to the higher temperatures. The turbidity of some *Arthrobacter* cultures decreased after prolonged incubation at 37°. Hagen & Rose (1961) also reported that cultures of the *Cryptococcus* which had stopped growing after they had been transferred to the higher temperature began to grow when they were transferred back to the optimum temperature for growth. There was usually a lag period before growth occurred at the optimum temperature, and this lag was proportional to the

duration of the incubation at the higher temperature. The results of subjecting each of the organisms used in the present work to a similar regimen of changes in incubation temperature are shown in Fig. 2. Cultures of each of the organisms grew after being transferred back to the optimum temperature for growth. The lag period for growth of the *Arthrobacter* and *Candida* increased with the length of time that the cultures had spent at the higher temperatures. But *Corynebacterium erythrogenes* cultures grew almost immediately after they were transferred back to the optimum temperature for growth.

It was possible that the inability, or limited ability, of the organisms to grow following the transfer of cultures to the higher temperatures was due to an additional nutritional demand that could not be met by the media (Brown, 1957). Several reports have appeared showing that, at temperatures above the maxima for growth in minimal medium, micro-organisms may become auxotrophic for growth factors that are not required at the optimum temperatures for growth (see review

Fig. 2. Growth of cultures (6 ml.) of *Arthrobacter* (a), *Candida* (b), *Corynebacterium erythrogenes* (c) after transfer from a temperature at or near the optimum for growth (●—●) to one 3–5° above the maximum for growth (○—○) followed by a return to the optimum temperature (----). Cultures of *Arthrobacter* were transferred to 37° after 120 hr at 20° and were returned to 20° after 24 (▲), 48 (△) and 175 (●) hr at 37°; cultures of *Candida* were transferred to 25° after 120 hr at 10°, and were returned to 10° after 12 (▲), 36 (△) and 60 (●) hr at 25°; *C. erythrogenes* cultures were transferred from 15° to 30° after 92 hr and were returned to 15° after 52 (▲), 100 (△) and 172 (●) hr at 30°.

by Langridge, 1963). Little attention has been given to the reasons for this increase in nutritional requirements with temperature, although it is assumed that they are caused in part at least by the thermal denaturation of one or more enzymes concerned in the synthesis of some cell constituent. To test for an increase in nutritional requirements at the higher temperatures, cultures of each of the three organisms were incubated at or near the optimum temperature for growth and, when the cultures had reached the mid-exponential phase, they were transferred to a higher temperature. At the time of transfer, duplicate cultures of each organism were supplemented with 0.5 ml. of a solution of bacteriological peptone ('Oxoid', Oxo Ltd., London, E.C. 4) to concentrations 1.0, 0.1 or 0.01 % (w/v), or of yeast extract ('Yeastrel', Brewers' Food Supply Co. Ltd., Edinburgh) to final concentrations 0.1 or 0.01 % (w/v). Other cultures were supplemented with peptone (to 1 %, w/v) + yeast extract (to 0.1 %, w/v). Control cultures were supplemented with water. However, none of these supplements had any detectable effect on the growth of the organisms at the higher temperatures. Although these results do not exclude the

possibility that transfer to the higher temperatures caused additional nutritional demands by the organisms, they show that such demands, if created, were not satisfied by the constituents of the bacteriological peptone and yeast extract. Neither of the bacteria grew in nutrient broth at the higher temperatures.

Effect of change in incubation temperature on viability and respiratory activity

From the report by Hagen & Rose (1962) it seemed likely that further information on the biochemical bases of the maximum temperatures for growth of the organisms might be obtained by examining the behaviour of organisms transferred to temperatures above the maxima for growth. The data in Fig. 3 show the effect

Fig. 3. Effect of change in incubation temperature on growth (●), viability (Δ) and the rate of respiration of endogenous reserves (◐) and of exogenous glucose (○) by *Arthrobacter* (a), *Candida* (b), *Corynebacterium erythrogenes* (c). Cultures (100 ml.) of *Arthrobacter* were transferred from 20° to 37° after 72 hr incubation; cultures of *Candida* from 10° to 25° after 120 hr; and *C. erythrogenes* cultures from 15° to 30° after 120 hr. — indicates the activities of organisms at the higher temperatures, and - - - the activities of organisms at the lower temperatures. Respiratory activities were measured at the temperatures at which the organisms had been incubated. Plate cultures for the estimation of viability were incubated at the lower temperature for each organism.

of such a change in incubation temperature on the viability and respiratory activity of each organism. The imposition of these thermal stresses caused a rapid decline in the rates of respiration of exogenous glucose and endogenous reserves by the *Arthrobacter* and the *Candida* and this was accompanied after a brief lag period by a marked decrease in the content of viable organisms in the cultures. *Corynebacterium erythrogenes*, on the other hand, was much less sensitive to the thermal stress. The rates of respiration of this bacterium were not so markedly affected after the transfer of cultures to 30°, while the content of viable organisms in these cultures actually increased slightly.

An attempt to locate the heat-sensitive lesions in the respiratory metabolism of the organisms was made by examining the ability of each organism to respire intermediates of the TCA cycle before and after transfer to the higher temperature.

Organisms were tested for the ability to respire pyruvate, citrate, isocitrate, 2-oxo-glutarate, succinate, fumarate, malate and oxaloacetate. Only a few of these substrates were respired to any appreciable extent by organisms grown at the optimum temperatures for growth; presumably those compounds not respired were unable to penetrate the organisms. Tucker (1960) examined the ability of a strain of *Corynebacterium erythrogenes* to respire TCA cycle intermediates after growth at

Fig. 4. Effect of change in incubation temperature on the ability of *Arthrobacter* (a), *Candida* (b), *Corynebacterium erythrogenes* (c) to respire endogenous reserves (●), glucose (○), pyruvate (△), succinate (●) and oxaloacetate (▲). Cultures (100 ml.) of *Arthrobacter* were transferred from 20° to 37° after 120 hr; cultures of *Candida* from 10° to 25° after 120 hr; and *C. erythrogenes* cultures from 15° to 30° after 120 hr. All respiratory activities were measured at the higher temperatures. Data are given only for those substrates that the organisms were able to oxidize before transfer to the higher temperatures.

25° in a medium containing glucose as carbon source, and obtained results similar to those reported here. The data in Fig. 4 show that the effects of the thermal stresses on the ability of the organisms to respire pyruvate were similar to the effects on glucose respiration. This suggested that one or more of the heat-sensitive lesions in each of the organisms was among the reactions of the TCA cycle, although it did not exclude the possibility that the ability of the organisms to transport the substrates had been affected by the change in incubation temperature. The abilities of *Arthrobacter* and *Candida* to respire succinate were similarly affected, which suggested that in these organisms there were heat-sensitive lesions among those reactions of the TCA cycle concerned with the oxidation of succinate. The effects of the increase in incubation temperature on the ability of *Corynebacterium erythrogenes* to respire pyruvate and oxaloacetate were also similar to the effect on glucose respiration. But the ability of this *Corynebacterium* to respire succinate was not adversely affected by the change in incubation temperature, which suggested that the enzymes concerned in the oxidation of succinate by it are not particularly heat-labile.

Activities of tricarboxylic acid cycle enzymes in organisms after changes in incubation temperature

The data in Table 1 show the effect of a change in incubation temperature on the activities of eight TCA cycle enzymes in each of the three organisms. The enzyme activities in extracts of the organisms that had been transferred to the higher temperatures were assayed at those temperatures and also at the temperatures at which the organisms had been grown before transfer. Control cultures were retained at the lower temperatures, and enzyme activities in extracts of organisms from those cultures were assayed only at those temperatures. Frequently there was some variation between experiments in the activities of enzymes in extracts of organisms. Nevertheless, it can be seen (Table 1) that the transfer of organisms from the optimum temperatures for growth to higher temperatures caused a marked decrease in the activities of many TCA cycle enzymes, particularly in *Arthrobacter* and *Candida*. With the exception of succinate dehydrogenase, the activities of each of the TCA cycle enzymes in *Arthrobacter* were diminished after cultures of the bacterium had been transferred to 37°. The diminution in activity was greatest with isocitrate dehydrogenase which was completely inactivated in bacteria that had been incubated at 37° for 48 hr. The activities of several of the enzymes in extracts of *Arthrobacter* that had been transferred to 37° were lower, when assayed at 20°, than the activities in organisms before they were transferred to 37°, which suggested that the loss of activity of these enzymes caused by the thermal stress was not completely reversible. However, the decrease in activity of malate dehydrogenase and to some extent of aconitate hydratase was reversed when the extracts were assayed at 20°. In *Candida*, the pyruvate dehydrogenase was most sensitive to the thermal stress although other enzymes (isocitrate dehydrogenase, fumarate hydratase) in this organism were not affected. The loss in activity of certain of the *Candida* enzymes was not completely reversible. The inactivation of malate dehydrogenase and aconitate hydratase was, however, reversed when the extracts were assayed at 10°. With *Corynebacterium erythrogenes*, the thermal stress had little if any effect on the activities of several of the enzymes, including pyruvate, 2-oxoglutarate and succinate dehydrogenases, and fumarate hydratase. Nevertheless, the activity of the isocitrate dehydrogenase in this bacterium was markedly decreased by the thermal stress.

Release of ultraviolet-absorbing compounds from organisms after changes in incubation temperature

That the lethal effect of high temperatures on micro-organisms may be caused partly by the breakdown of nucleic acids was first suggested by Califano (1952), Strange & Shon (1964) reported that heat-accelerated death of *Aerobacter aerogenes* in non-growing suspensions at 47° was accompanied by the degradation of endogenous RNA which led to an increase in the ultraviolet (u.v.)-absorption of cold acid extracts of the bacterium and of the suspending fluid. It seemed of interest, therefore, to examine the u.v.-absorption of cold acid extracts and culture filtrates of the organisms used in the present work, after the transfer of cultures to high temperatures, to assess the extent to which RNA degradation occurred. The results (Table 2) showed that the transfer of *Candida* cultures from 10° to 25° and of *Coryne-*

Table 1. *Effect of change in incubation temperature on the activities of tricarboxylic acid cycle enzymes in three micro-organisms*

Arthrobacter cultures were transferred to 37° after 120 hr at 20°; Candida cultures to 25° after 144 hr at 10°; *Corynebacterium erythrogenes* cultures to 30° after 96 hr at 15°. Control cultures were maintained at the lower temperatures. After the times indicated, cultures (100 ml.) of each organism were removed, and the organisms harvested and washed by centrifugation at 0°. The methods used for preparing cell extracts and assaying enzyme activities in the extracts are as described under Methods.

Enzyme	Arthrobacter						Candida						<i>Corynebacterium erythrogenes</i>					
	Temperature of			Incubation (hr)			Temperature of			Incubation (hr)			Temperature of			Incubation (hr)		
	I*	A†		Specific activity			I*	A†		Specific activity			I*	A†		Specific activity		
Pyruvate dehydrogenase	20°	20°		0.5	0.4	0.7	10°	10°		0.3	—	0.6	15°	15°		0.4	—	0.4
	37°	20°		0.5	0.6	0.2	25°	10°		0.5	0.1	0.0	30°	15°		0.4	0.5	0.2
	37°	37°		0.9	1.2	0.3	25°	25°		1.3	0.3	0.0	30°	30°		0.5	0.6	0.5
Citrate synthase	20°	20°		1.3	0.9	0.6	10°	10°		4.2	—	4.1	15°	15°		2.6	5.7	4.3
	37°	20°		1.3	0.6	0.3	25°	10°		5.6	7.1	4.9	30°	15°		2.6	2.8	3.4
	37°	37°		1.7	0.7	0.3	25°	25°		10.7	10.8	8.8	30°	30°		2.4	1.8	2.0
Aconitate hydratase	20°	20°		4.3	4.9	6.3	10°	10°		0.8	—	1.8	15°	15°		2.2	2.4	3.9
	37°	20°		7.2	5.1	4.7	25°	10°		0.2	0.2	0.2	30°	15°		5.0	3.6	3.4
	37°	37°		21.5	15.0	7.4	25°	25°		0.8	0.5	0.3	30°	30°		11.8	13.2	8.9
Isocitrate dehydrogenase	20°	20°		3.7	2.7	3.7	10°	10°		0.5	—	0.5	15°	15°		2.4	4.8	4.3
	37°	20°		4.3	0.5	0.5	25°	10°		0.5	0.7	0.4	30°	15°		2.1	0.7	0.2
	37°	37°		3.7	1.3	0.0	25°	25°		1.2	1.2	1.1	30°	30°		7.0	3.1	1.8
2-Oxoglutarate dehydrogenase	20°	20°		0.7	0.3	0.3	10°	10°		0.7	—	0.8	15°	15°		0.6	0.3	0.5
	37°	20°		0.6	0.4	0.1	25°	10°		0.4	0.4	0.4	30°	15°		0.5	0.6	0.3
	37°	37°		1.1	0.5	0.2	25°	25°		0.5	0.5	0.1	30°	30°		0.6	1.0	0.6
Succinate dehydrogenase	20°	20°		0.4	0.6	0.4	10°	10°		0.7	—	0.8	15°	15°		0.9	0.9	0.6
	37°	20°		0.9	0.9	0.8	25°	10°		0.5	0.5	0.4	30°	15°		0.6	0.6	0.6
	37°	37°		1.8	2.8	2.6	25°	25°		0.5	0.9	0.4	30°	30°		1.5	1.3	1.4
Fumarate hydratase	20°	20°		25.2	22.8	41.6	10°	10°		18.0	—	16.8	15°	15°		14.4	13.2	9.6
	37°	20°		29.2	32.0	20.0	25°	10°		8.8	9.6	8.0	30°	15°		9.6	10.0	8.4
	37°	37°		61.2	50.4	45.2	25°	25°		20.4	18.4	45.4	30°	30°		5.6	7.6	9.6
Malate dehydrogenase	20°	20°		4.5	4.7	7.8	10°	10°		2.1	—	2.8	15°	15°		2.1	2.7	4.7
	37°	20°		2.9	2.1	3.0	25°	10°		2.2	3.8	2.3	30°	15°		6.9	4.2	3.3
	37°	37°		5.3	3.0	4.9	25°	25°		6.1	2.9	3.9	30°	30°		12.4	10.2	9.7

* I = incubation.

† A = assay.

bacterium erythrogenes cultures from 15 to 30° did not lead to any additional intracellular accumulation or excretion of u.v.-absorbing compounds. There was, however, an increase in the u.v. absorption of filtrates from the cultures of *Arthrobacter* which had been transferred to 37°, although this was not accompanied by an increased intracellular accumulation of these compounds.

Table 2. *Effect of change in incubation temperature on the contents of ultraviolet-absorbing compounds in culture filtrates and cell extracts of the three organisms*

Arthrobacter cultures were transferred to 37° after 144 hr at 20°; *Candida* cultures to 25° after 168 hr at 10°; *Corynebacterium erythrogenes* cultures to 30° after 120 hr at 15°; control cultures were maintained at the lower temperatures. After the times indicated, duplicate cultures (100 ml.) of each organism were removed and the organisms harvested by centrifugation at the temperature at which they had been incubated. Portions of each culture filtrate were filtered through a Millipore filter with 25 mm. HA filters. The extinctions of filtrates from *Candida* cultures were measured at 260 m μ in quartz cuvettes with 1 cm. light path, but because of interference from amino acids filtrates from *Arthrobacter* and *C. erythrogenes* cultures were measured at 300 m μ . The crops of organisms were washed twice with phosphate buffer (pH 7.0 for bacteria; pH 4.5 for yeast), a portion of suspension containing equiv. 5 mg. dry wt. organism taken and the organisms extracted with 3 ml. 5% (w/v) trichloroacetic acid at room temperature. The supernatant fluids were quickly removed by centrifugation and made to 5.0 ml. with 5% trichloroacetic acid. The extinctions of these extracts were measured at 260 m μ in 1 cm. quartz cuvettes.

Organism	Period of incubation after transfer (hr)	Cultures maintained at optimum temperature			Cultures transferred to temperature above maximum for growth		
		Ultraviolet absorption of		Growth (mg. dry wt./ml.)	Ultraviolet absorption of		Growth (mg. dry wt./ml.)
		Cell extract ($E_{260\text{ m}\mu}^{1\text{ cm.}}$)	Filtrate ($E_{300\text{ m}\mu}^{1\text{ cm.}}$)		Cell extract ($E_{260\text{ m}\mu}^{1\text{ cm.}}$)	Filtrate ($E_{260\text{ m}\mu}^{1\text{ cm.}}$)	
<i>Arthrobacter</i>	0	0.81	0.08	0.26	—	—	—
	24	0.81	0.10	0.31	0.04	0.19	0.26
	48	0.22	0.08	0.42	0.31	0.48	0.25
	72	0.24	0.12	0.42	0.25	0.63	0.24
<i>Candida</i>	0	0.07	0.08*	0.20	—	—	—
	5	0.09	0.07*	0.21	0.11	0.11*	0.23
	24	0.07	0.12*	0.23	0.03	0.17*	0.25
	48	0.07	0.17*	0.27	0.04	0.16*	0.25
<i>Corynebacterium erythrogenes</i>	0	0.20	0.06	0.11	—	—	—
	24	0.23	0.0	0.13	0.26	0.0	0.13
	48	0.10	0.0	0.18	0.22	0.01	0.18

* Extinction measured at 260 m μ

DISCUSSION

In the work described in the present paper, we were concerned only with two of the biochemical factors that seemed likely to be involved in determining the maximum temperatures for growth of the organisms, namely, enzyme denaturation and nucleic acid breakdown; no attempt was made to examine the effect of thermal stress on the properties of membrane lipids in the micro-organisms. Moreover, since we set out to study the biochemical bases of the maximum temperatures for growth

of these organisms, all experiments in which the organisms were subjected to a thermal stress were done with cultures of organisms rather than with washed suspensions or cell extracts, the conditions in which are known to alter the susceptibility of certain microbial cell constituents to heat inactivation (Militzer & Burns, 1954; Strange & Shon, 1964).

The rapid decrease in the respiratory activities of the *Arthrobacter* and *Candida*, caused by transferring exponentially growing cultures to a temperature 3–5° above the maximum for growth in freshly inoculated culture, suggested that inactivation of the respiratory metabolism is a major factor in determining the maximum temperature for growth of these organisms. Such an inactivation might lead quickly to a shortage of metabolic energy in the organisms, and might explain the increased death rate which accompanied the decline in respiratory activity. The shortage of metabolic energy might also explain in part the inability of organisms to grow at the higher temperatures in media supplemented with bacteriological peptone and/or yeast extract.

The activities of several TCA cycle enzymes in the *Arthrobacter* and *Candida* were diminished after these organisms had been transferred to the higher temperatures. The isocitrate dehydrogenase in the *Arthrobacter* and the pyruvate dehydrogenase in the *Candida* were almost completely inactivated in organisms so transferred and this might explain the almost complete loss of respiratory activity by these organisms at the higher temperatures. However, the inactivation of other respiratory enzymes, such as those concerned in oxidative phosphorylation, might also contribute to this effect. Other workers have reported exceptionally heat-labile respiratory enzymes in psychrophilic micro-organisms. Upadhyay & Stokes (1963) showed that the formate hydrogenlyase in a psychrophilic bacterium was inactivated at a much lower temperature than was the corresponding enzyme in a mesophilic strain of *Escherichia coli*; and Burton & Morita (1963) and Morita & Burton (1963) reported that the malate dehydrogenase in another psychrophilic bacterium was inactivated at 30°, which is the maximum temperature for growth of this bacterium. However there has been no previous report of a comparison of the heat lability of several respiratory enzymes in any one psychrophilic organism. Burton & Morita (1963) reported that the denaturation of the malate dehydrogenase in extracts of their bacterium was to some extent reversible, and suggested that the bacterium might contain more than one malate dehydrogenase, each with a different heat lability. Certain of our results may also be explained by postulating the existence of isoenzymes with different degrees of heat lability. There is also the possibility that the thermal stresses affected not only the activities of certain of the TCA cycle enzymes but also the ability of the organisms to synthesize these enzymes.

The transfer of cultures of *Corynebacterium erythrogenes* from the optimum temperature for growth to the higher temperature also caused a decline in the respiratory activity of the organism, but this was much less marked than with *Arthrobacter* and *Candida* and was not accompanied by a decrease in viability. The lack of an effect on the viability explains why *C. erythrogenes*, which had been grown at 15° and then transferred to 30°, grew rapidly on being returned to 15°. The comparatively small effect of the thermal stress on the respiratory activity of *C. erythrogenes* was supported by the finding that the activities of several of the TCA cycle enzymes in extracts of this bacterium were not diminished after the transfer

of the bacteria from 15° to 30°. The most heat-sensitive enzyme in this corynebacterium was isocitrate dehydrogenase and the decline in activity of this enzyme was of the same order as the decrease in respiratory activity. Possibly therefore the bacterium can produce sufficient energy at the higher temperature to maintain viability but insufficient to enable the organisms to divide.

Breakdown of RNA, as detected by an increase in the u.v.-absorption of cold acid extracts of the organisms and culture filtrates, did not appear to be important in determining the maximum temperatures for growth of any of the organisms tested. There was some excretion of u.v.-absorbing compounds by *Arthrobacter* after transfer to 37°, but the amounts released were small and probably did not represent an appreciable loss of RNA. Strange & Shon (1964) showed that magnesium ion protected RNA in *Aerobacter aerogenes* against thermal denaturation; it is possible that the thermal decomposition of RNA in the organisms used in the present study was protected by Mg ions present in the medium.

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