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Heat-labile Enzymes in a Psychrophilic Bacterium

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ABSTRACT

The oxidative and fermentative activities of a psychrophilic bacterium (strain 82), whose maximal growth temperature is 35 C, were completely destroyed by exposure of the cells to 46 C for about 1 hr, whereas those of mesophilic *Escherichia coli* were unaffected. Similar results were obtained with cell-free extracts. In attempts to determine some of the specific enzymes inactivated in strain 82 by exposure to 46 C, it was found that reduced nicotinamide adenine dinucleotide oxidase was completely inactivated at 46 C in 2 hr. Also, cytochrome *c* reductase was completely destroyed at 46 C in 1 hr and was 70% destroyed at 40 C in 2 hr. The heat lability of the latter may determine the maximal growth temperature of the organism. In addition, the results indicated that the enzymes of strain 82 involved in the clastic split of pyruvate to formate and acetate are inactivated by exposure to 46 C and that the lactic and glycerol dehydrogenases are more heat-labile than those in *E. coli*. Succinic, nicotinamide adenine dinucleotide phosphate-alcohol, and glucose-6-phosphate dehydrogenases, however, in both strain 82 and *E. coli*, were essentially unaffected by exposure to 46 C for 2 hr.

Psychrophilic and mesophilic microorganisms differ markedly in their cardinal growth temperatures. Psychrophiles can grow at 0 C and lower temperatures, compared to a minimum of about 10 C for mesophiles. Moreover, the maximal growth temperature of most psychrophiles is approximately 25 to 30 C compared, to 35 to 50 C for most mesophiles (13). The available evidence suggests that the lower maximal growth temperatures of psychrophiles may be due to the relatively greater heat lability of some of their enzymes (3, 6, 10, 18).

In previous investigations of a gram-negative, rod-shaped, motile, psychrophilic bacterium, designated as strain 82, it has been shown that both formic hydrogenlyase (21) and hydrogenase (22) in this psychrophile are much more heat-sensitive than are the corresponding enzymes in *Escherichia coli* and other mesophilic bacteria. In the present investigation, a search was made for additional heat-sensitive enzymes in strain 82. Some have been found and are described in this paper.

MATERIALS AND METHODS

The psychrophilic bacterium used, strain 82, has morphological and physiological properties which resemble those of enterobacteria (21). It can grow at 0 C and has a maximal growth temperature of 35 C. For comparative purposes, a mesophilic strain of *E. coli* with a maximal growth temperature of 45 C was employed. The growth medium consisted of 2% Trypticase and 1% glycerol in distilled water and was adjusted to *p*H 7.1. The stock cultures were maintained on agar slants of the same medium.

All suspensions for manometric and other experiments were prepared in the following manner. The cells from a slant culture of strain 82 or *E. coli* grown for 1 day at 20 or 30 C, respectively, were suspended in 4 ml of Trypticase-glycerol broth (basal medium). We used 0.8 ml of this suspension to inoculate 400 ml of the basal medium contained in a 2-liter Erlenmeyer flask. Occasionally, 1-liter flasks containing 200 ml of medium were used and the size of the inoculum was decreased proportionally. The broth cultures of both organisms were incubated at 30 C for about 14 hr with vigorous agitation on a rotary shaker. At this time, the cultures were near the end of the exponential phase of growth and had turbidities of about 310 and 420 units for strain 82 and *E. coli*, respectively, when measured in a Klett-Summerson photoelectric colorimeter (660-mμ filter). The cells were harvested by centrifugation at 4 C, washed twice with 0.1 M K_2HPO_4—KH_2PO_4 buffer (*p*H 7.0), and finally resuspended in sufficient buffer to give a turbidity of 450 Klett units. Such suspensions contained 2.7 mg of strain 82 and 2.5 mg of *E. coli* per ml on a dry weight basis.

Cell-free extracts were prepared by passing washed cells suspended in 0.1 M phosphate buffer (*p*H 7.0) at 20-fold the usual cell concentration of 450 Klett units through a French pressure cell at 4 C and about 7,000 psi. The disrupted cell suspensions were used without further treatment. Microscopic examinations revealed only one or two intact cells per field.

For the manometric oxidation experiments, each Warburg vessel received 2.0 ml of cell suspension in the main compartment, 4 μmoles of substrate dissolved in 0.1 ml of water in the side arm, and 0.2 ml of 20% KOH in the center well to absorb CO_2. The bath temperature was 30 C, and the gas phase was air unless otherwise indicated. A similar procedure was used to measure the rate of O_2 consumption by cell-free extracts. The fermentation of glucose was determined by measuring the rate of CO_2 liberation from bicarbonate due to acid formation under anaerobic conditions (20).

To determine the effect of heat on oxidative, fermentative, and other enzyme activities, whole and disrupted cell preparations were heated at 46 C in screw-capped tubes in a water bath for various periods of time and then cooled in ice water.

Glucose concentrations were determined manometrically, according to the procedure of Bentley (2), with glucose aerodehydrogenase containing catalase, at *p*H 6.6 in 0.1 M potassium phosphate buffer. With whole-cell suspensions, the cells were first removed by centrifugation, and a portion of the supernatant fluid was used for the glucose determination. In the case of cell-free preparations, portions were heated in a boiling-water bath for about 3 min to stop metabolism prior to determining the amount of glucose present.

Pyruvate was determined according to the method of Friedemann and Haugen (9) after deproteinization with 10% trichloroacetic acid. The determination of L(+)-lactate was carried out according to the procedure of Hohorst (12) after deproteinization with 0.5 M perchloric acid. Lactic acid was measured chemically by the methods of Friedemann and Graeser (8) and Barker and Summerson (1). Formic acid was estimated by oxidation with $HgCl_2$ in slightly acid acetate buffer and weighing the mercurous chloride formed (7). Acetic acid was determined by gas chromatography (11) with a Beckman GC2 gas chromatograph equipped with a hydrogen flame detector. A Carbowax (20-mesh) 36 inch × 0.25 inch column was used. The sample was acidified with metaphosphoric acid to *p*H 2.5, and 10 μliters of the sample was injected. The concentration of acetic acid was calculated by comparing the area under the curve with that of a known concentration of acetic acid.

Succinic dehydrogenase was determined manometrically with phenazine-methosulfate as artificial electron acceptor (19). The assays were carried out at 30 C in 0.1 M phosphate buffer (*p*H 7.6) with an excess of the electron acceptor.

Cell-free extracts for the determination of reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase and other dehydrogenases were prepared as follows. Twenty-fold concentrated cell suspensions were disrupted in a French press and centrifuged at 18,800 × *g* for 30 min to remove cellular debris. The supernatant liquid was dialyzed against three changes of glass-distilled water, 2 liters each, for 12 hr and then was dialyzed against 2 liters of 0.05 M potassium phosphate buffer (*p*H 7.5) for another 12 hr. During dialysis, water and buffer were constantly agitated with a magnetic stirrer. The dialysate was then centrifuged at 105,000 × *g* for 60 min in a Spinco model L preparative ultracentrifuge. The supernatant fluid, after dilution with the above phosphate buffer, was used for the dehydrogenase determinations. All of the above operations were carried out at temperatures below 4 C.

Cell-free extracts for cytochrome *c* reductase (diaphorase) determinations were prepared as for the dehydrogenase assays, but with the following modifications. Dialysis against distilled water was carried out for about 20 hr, and then the dialysate was centrifuged at 18,800 × *g* for 30 min. The supernatant liquid, after dilution with 0.02 M tris(hydroxymethyl-aminomethane (Tris)-chloride buffer (*p*H 7.5), was used for the enzyme assays.

Protein was determined by the method of Lowry et al. (14) with crystalline bovine serum albumin as the standard.

All spectrophotometric measurements of enzyme activity were made with a Beckman DU spectrophotometer equipped with a Gilford absorbance indicator and automatic cuvette positioner attached to a Honeywell variable-speed chart recording unit. All assays were made at room temperature with 0.6- or 3-ml silica cuvettes. The $NADH_2$ oxidase, lactic dehydrogenase, and glycerol dehydrogenase were measured by the decrease in absorbance at 340 mμ due to oxidation of $NADH_2$, with oxygen, pyruvate, and dihydroxyacetone, respectively, as hydride acceptors. Nicotinamide adenine dinucleotide phosphate (NADP)-alcohol dehydrogenase and glucose-6-phosphate dehydrogenase were assayed by the methods of DeMoss (4, 5). The specific conditions for the enzyme determinations are given with each enzyme assay.

All experiments were repeated several times and representative data are presented.

RESULTS

Effect of heating cells at 46 C on substrate oxidation and fermentation. Whole-cell suspensions of psychrophilic strain 82 and mesophilic *E. coli* readily oxidized glucose, glucosamine, glycerol, and pyruvate. The effect of heating cell suspensions of both organisms at 46 C for various periods of time on the oxidation of these substrates was determined. This temperature was chosen because it is relatively close to the maximal growth temperature of 35 C for strain 82, and in preliminary experiments, was found to inactivate the metabolism of the organism within an experimentally convenient period of time of about 1 hr. The effects of heating on the oxidation of glucose by strain 82 and *E. coli* are shown in Fig. 1.

With the psychrophile there was a marked reduction in oxidative activity after only 10 min of heating. Most of the activity was abolished in 30 min and all of it in 60 min. In marked contrast, there was virtually no reduction of the oxidative activity of *E. coli* even after 120 min of heating. Similar results were obtained for the oxidation of glucosamine, glycerol, and pyruvate. Heating at 46 C destroyed the ability of strain 82 to oxidize

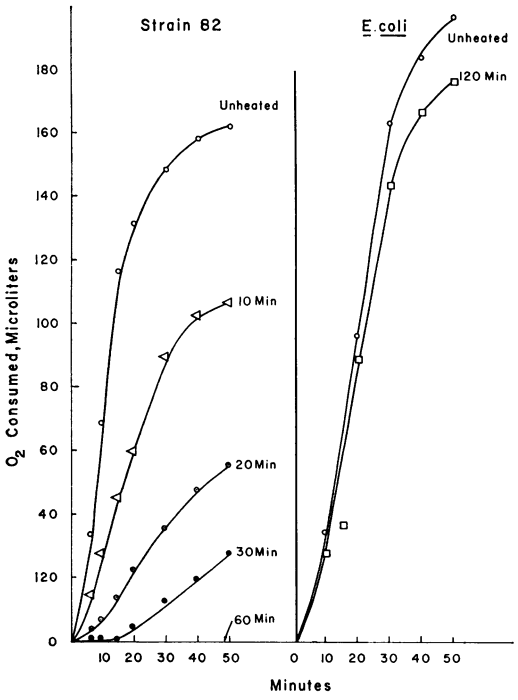

FIG. 1. *Effect of heating cell suspensions of strain 82 and Escherichia coli at 46 C on the oxidation of glucose. Endogenous oxidation has been subtracted.*

these compounds but had little or no effect on the oxidative activity of *E. coli*. Not all of the enzymes of the psychrophile are equally heat-labile. The complete elimination of glucose oxidation required a 60-min heating period, whereas only 20 and 45 min, respectively, were required to abolish completely oxidation of glycerol and pyruvate.

Because of the possibility that elimination of glucose oxidation in strain 82 by heat may involve substrate permeability rather than destruction of internal glucose-oxidizing enzymes, the effect of high concentrations of glucose was determined. At high concentrations, glucose was able to enter the heated cells passively, by diffusion. The data in Table 1 show that the rate of glucose oxidation by unheated cells of strain 82 was unaltered by increasing the glucose level from 10 to 400 μmoles per vessel. Also, chemical analyses indicated that all or some of the glucose disappeared from the suspensions during oxidation. The cells which had been heated at 46 C for 120 min, however, failed to oxidize glucose at all glucose levels, and all of the added glucose was still present at the termination of the experiment. These results suggest that heating of strain 82 denatured at least those enzymes involved in the initial stages of the glucose-oxidation process.

To determine the effect of heat on the fermentation of glucose, the cell suspensions in phosphate buffer were heated at 46 C for 15 and 30 min and cooled in ice water. The heated cells were recovered by centrifugation, resuspended in an equivalent amount of 0.01 M $NaHCO_3$, and tested manometrically for their ability to ferment glucose. The gas phase was a mixture of 95% N_2 and 5% CO_2 freed of O_2 by passage over hot copper turnings. The evolution of CO_2, formed by the interaction of the bicarbonate buffer with the organic acids produced in the fermentation of glucose, was followed. The cells did not contain formic hydrogenlyase because of the conditions under which they were grown, and therefore metabolic CO_2 was not produced.

As shown in Fig. 2, the fermentation of glucose by strain 82 also is heat-labile. Exposure of the

TABLE 1. *Influence of substrate concentration on rate of glucose oxidation by unheated and heated cells of strain 82*

Amt of glucose per vessel	Unheated		Heated[a]	
	Q_{O_2}[b]	Residual glucose	Q_{O_2}[b]	Residual glucose
μmoles		μmoles		μmoles
10	127	0	0	9.9
100	125	71	0	101
200	128	177	0	212
400	128	360	0	410

[a] At 46 C for 120 min.
[b] Endogenous oxidation subtracted.

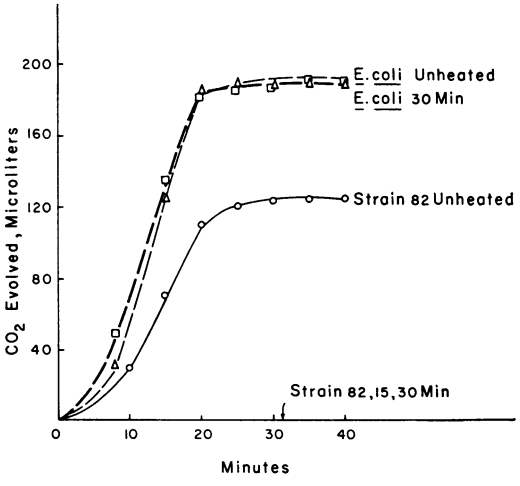

FIG. 2. *Effect of heating cell suspensions of strain 82 and Escherichia coli at 46 C on the fermentation of glucose.*

psychrophile cells to 46 C for only 15 min completely destroyed their fermentative activity. In contrast, glucose fermentation by mesophilic *E. coli* was unimpaired by exposure of the cells to 46 C for 30 min.

Effect of heating of cell-free extracts of strain 82 on oxidation and fermentation. To obtain further information on the effect of heat on the enzymes of psychrophilic strain 82, without the possible complicating factor of permeability, experiments were made with cell-free extracts. The extracts and whole cells used as controls were both heated at 46 C for 120 min, and the substrate level was raised to 100 μmoles per manometric vessel. Disruption of the cells resulted in considerable loss of oxidative activity. To obtain activities comparable to those of whole cells, it was necessary to use the cell-free preparations at a concentration equivalent to 16 times that of the whole cells.

The comparative effects of heating whole and disrupted cells of strain 82 on glucose oxidation are shown in Fig. 3. Both whole and disrupted cells rapidly oxidized glucose. However, glucose oxidation was completely abolished with both types of cell preparation after heating them at 46 C for 120 min. Thus, the heat treatment appeared to destroy enzymes involved in the oxidation of glucose.

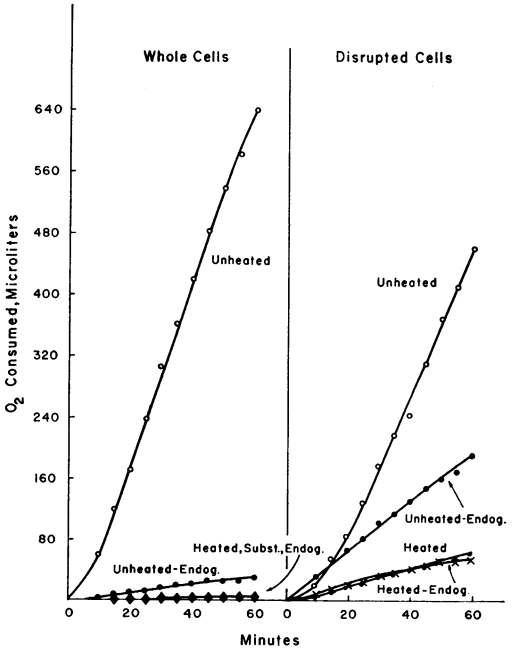

FIG. 3. *Effect of heating whole cells and cell-free extracts of strain 82 on oxidation of glucose. Each vessel received 2.0 ml of whole cells, equivalent to 5.4 mg of cells (dry weight), or 1.6 ml of disrupted cells, equivalent to 86.4 mg of whole cells (dry weight), 0.4 ml of phosphate buffer, 100 μmoles of glucose in 0.1 ml of water, and 0.2 ml of 20% KOH in the center well to absorb CO_2. The gas phase was air, and the bath temperature was 30 C.*

Similar results were obtained for the oxidation of pyruvate (Fig. 4). Heating at 46 C for 120 min eliminated pyruvate oxidation by whole cells and cell-free extracts of strain 82.

Chemical analyses for residual glucose and pyruvate in experiments similar to those described above confirmed the manometric O_2 consumption data. Unheated whole and disrupted cells consumed glucose and pyruvate, and heated whole cells did not metabolize these substrates. However, heated cell-free extracts of strain 82 converted glucose to pyruvate, and 0.5 to 1.0 μmole of pyruvate was obtained per μmole of utilized glucose. L (+)-Lactic acid was not formed. Pyruvate was not metabolized by the heated cell-free preparations. It is not clear why heated whole cells do not attack glucose, but heated cell-free extracts convert glucose to pyruvate.

Pyruvate is also fermented by unheated whole cells and cell-free extracts of strain 82, as shown by the disappearance of pyruvate when added to both types of cell preparations under anaerobic conditions. With whole cells, about 70% of the pyruvate was fermented to equimolar quantities of formic and acetic acids. However, heating of the cell preparations at 46 C for 120 min stopped the fermentation of pyruvate. These results suggest that the enzymes involved in the clastic split of pyruvate are heat-labile.

Effect of heat on succinate oxidation and succinic dehydrogenase activity. Succinate is oxidized by both whole cells and cell-free extracts of strain 82 (Fig. 5 and 6). Heating of both cell preparations at 46 C for 120 min or less abolished succinate oxidation. In contrast, oxidation of succinate by *E. coli* is unaffected.

To determine whether succinic dehydrogenase was the heat-sensitive link in succinate oxidation, assays for the enzyme were made before and after heating the cell-free extracts. The manometric phenazine methosulfate method was used. The results are shown in Fig. 6. The succinic dehydrogenase activity of strain 82 was unaffected by the heat treatment, and therefore the heat-sensitive point in succinate oxidation must be some other enzymatic component of the electron transport system.

Heat stability of other dehydrogenases. These included the nicotinamide adenine dinucleotide (NAD)- and NADP-linked dehydrogenases, $NADH_2$ oxidase, and lactic, glycerol, alcohol, and

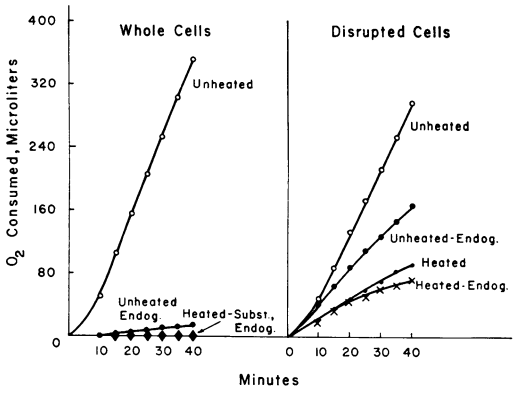

FIG. 4. *Effect of heating whole cells and cell-free extracts of strain 82 on oxidation of pyruvate. The experimental conditions were the same as for glucose oxidation (Fig. 3). Endogenous oxidation has been subtracted.*

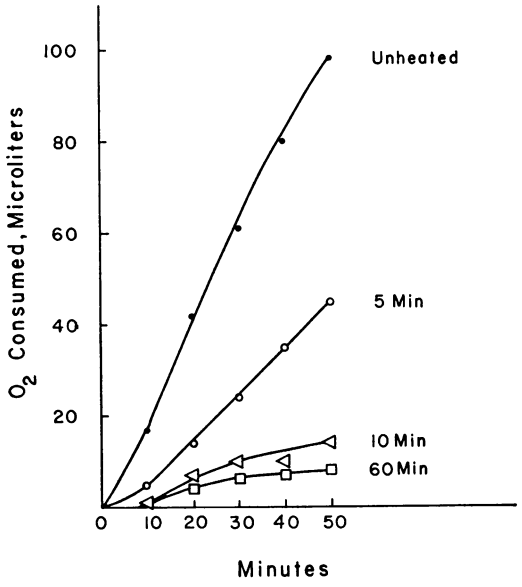

FIG. 5. *Effect of time of heating at 46 C on oxidation of 10 μmoles of succinate by intact cells of strain 82.*

glucose-6-phosphate dehydrogenase. The effects of heating cell-free extracts of strain 82 and *E. coli* at 46 C for various periods of time on the activities of the dehydrogenases are shown in Table 2.

About 90% of the $NADH_2$ oxidase activity of strain 82 was destroyed in 30 min of heating, and all of it, in 120 min. With *E. coli*, 30% of $NADH_2$ oxidase activity was destroyed in the first 30 min, and there was no further destruction on heating for an additional 90 min. Lactic dehydrogenase was more heat-stable than was $NADH_2$ oxidase in strain 82, although approximately two-thirds of the activity in strain 82 and approximately one-half in *E. coli* was eliminated in 120 min. Glycerol dehydrogenase activity in strain 82 decreased 35% after 30 min of heating, but there was no further destruction on continued heating. With *E. coli*, only 10% of the activity was eliminated within the first 30 min, and about 40%, after 120 min. Dehydrogenases of NADP-alcohol and glucose-6-phosphate in both organisms were essentially unchanged in activity after 60 or 120 min of heating. These dehydrogenases, like succinic dehydrogenase, are stable at 46 C.

Heat stability of cytochrome c reductase. The previous results indicated that the $NADH_2$ oxidase system in strain 82 was especially sensitive to heating at 46 C. Since this enzyme system includes cytochrome *c* reductase (diaphorase), the heat stability of the latter at 46 C was determined in cell-free extracts of both organisms. Mahler et al. (16) consider that pig heart $NADH_2$ cytochrome *c* reductase and diaphorase activities are due to the same enzyme molecule. Assay for cytochrome *c* reductase was made by the procedure of Mahler (15).

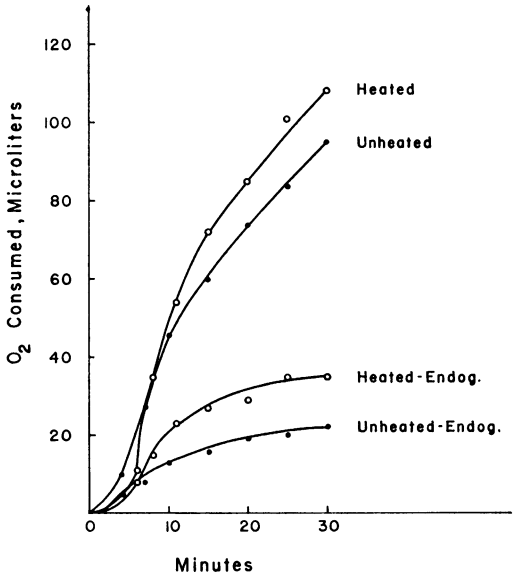

FIG. 6. *Effect of heating at 46 C for 120 min on the succinic dehydrogenase activity of cell-free extracts of strain 82. Each Warburg vessel received 0.5 ml of cell extract, equivalent to 27 mg of cells (dry weight), 1.5 ml of 0.1 M potassium phosphate buffer (pH 7.6), 2.0 mg of phenazine methosulfate in 0.2 ml of water, 30 μmoles of KCN (approximate pH 7.0) in 0.3 ml of water, and 0.3 ml of 0.2 M sodium succinate (60 μmoles). The gas phase was air, and the bath temperature was 30 C.*

TABLE 2. *Effect of heating on dehydrogenases of strain 82 and Escherichia coli*

Organism	Period of heating at 46 C	Specific activity[a]				
		$NADH_2$ oxidase[b]	Lactic dehydrogenase[c]	Glycerol dehydrogenase[d]	NADP-alcohol dehydrogenase[e]	Glucose-6-phosphate dehydrogenase[f]
	min					
Strain 82	0	0.38	0.99	1.50	0.085	2.50
	30	0.04	0.82	0.97	0.087	2.50
	60	0.02	0.68	0.99	0.087	2.50
	120	0.00	0.34	0.99	0.078	2.18
E. coli	0	0.33	2.10	1.61	0.117	2.27
	30	0.23	1.70	1.45	0.110	2.27
	60	0.23	1.40	1.16	0.111	2.27
	120	0.23	1.00	0.95	0.103	2.17

[a] Change in absorbance at 340 mμ per minute per mg of protein at room temperature.

[b] Complete system consisted of 2.7 ml of 0.05 M potassium phosphate buffer (*p*H 7.5), and 1 μmole of $NADH_2$ dissolved in 0.1 ml of 0.05 M phosphate buffer (*p*H 7.5). The reaction was started by adding 0.32 mg (0.025 ml) and 0.09 mg (0.025 ml) of protein of strain 82 and *E. coli*, respectively. The final volume was 2.825 ml. The blank cuvette contained all the components except protein solution.

[c] Complete system consisted of 2.6 ml of 0.05 M potassium phosphate buffer (*p*H 7.5), 1 μmole of $NADH_2$ in 0.1 ml of 0.1 M phosphate buffer (*p*H 7.5), and 10 μmoles of sodium pyruvate dissolved in 0.1 ml of water. The reaction was started by adding 0.32 mg (0.025 ml) and 0.09 mg (0.025 ml) of protein of strain 82 and *E. coli*, respectively. The final volume was 2.825 ml. The blank cuvette contained all of the components except pyruvate. For calculating specific activities, the decrease in absorbance due to endogenous oxidation of $NADH_2$ was subtracted.

[d] Complete system consisted of 0.5 ml of 0.05 M potassium phosphate buffer (*p*H 7.5), 0.02 ml of 0.1 M dihydroxyacetone, 0.02 ml of 0.01 M $NADH_2$, 0.08 mg of cell protein contained in 0.02 ml of phosphate buffer (*p*H 7.5), and 0.04 ml of water. The final volume was 0.6 ml, and the reaction was started by the addition of protein. The blank cuvette contained all of the components except dihydroxyacetone. The specific activities were calculated after subtracting the decrease in absorbance due to endogenous oxidation of $NADH_2$.

[e] Complete system consisted of 0.4 mg of cell protein (0.1 ml), 0.04 ml of 0.0024 M NADP, 0.3 ml of 0.2 M Tris-chloride buffer (*p*H 7.5), 0.06 ml of 5 M ethyl alcohol, and 0.1 ml of water. The final volume was 0.6 ml. The reaction was started by adding ethyl alcohol. The blank cuvette without alcohol did not show any reduction of NADP.

[f] Complete system consisted of 0.04 mg of cell protein (0.01 ml), 0.3 ml of 0.1 M Tris-chloride buffer (*p*H 7.8), 0.02 ml of 0.1 M $MgCl_2$, and 0.04 ml of 0.0027 M NADP. The reaction was started by adding 0.05 ml of 0.04 M glucose-6-phosphate. The final volume was 0.6 ml. The blank cuvette contained all of the components except glucose-6-phosphate. There was no increase in absorbance in the blank cuvette.

As shown in Fig. 7, strain 82 lost 87% of its cytochrome *c* reductase activity during 30 min of exposure to 46 C and lost all of it in 60 min, whereas that of *E. coli* was essentially unchanged. The possibility that the loss in cytochrome *c* reductase activity on heating is due to liberation of an enzyme inhibitor seems unlikely, since activity could be restored in the heated cell-free extract by the addition of unheated extract. Therefore, the heat lability of the $NADH_2$ oxidase system in strain 82 may be due to the heat lability of the cytochrome *c* reductase component of the electron transport chain.

Because of the marked heat lability of cytochrome *c* reductase in strain 82 at 46 C, lower heating temperatures were tested. Cell-free extracts of both organisms were diluted with 0.02 M Tris-chloride buffer (*p*H 7.5) to contain 4.0 mg of protein per ml. Portions of 1 ml were heated at 37, 40, and 45 C for 120 min, cooled in ice water, and assayed for cytochrome *c* reductase activity. Unheated cell-free extracts, maintained at 0 C, were included in the assays. The results are given in Table 3. No reduction in activity of strain 82 occurred at 37 C in 120 min, but there was a 70% reduction in activity at 40 C and complete inactivation at 45 C. In *E. coli*, enzyme activity was stable at 37 and 40 C, but it showed a small decrease at 45 C.

The relatively rapid and extensive inactivation of cytochrome *c* reductase in strain 82 at 40 C may be particularly significant since the organism has a maximal growth temperature of 35 C. Inactivation of this enzyme may be at least one of the factors which prevents the organism from growing above 35 C.

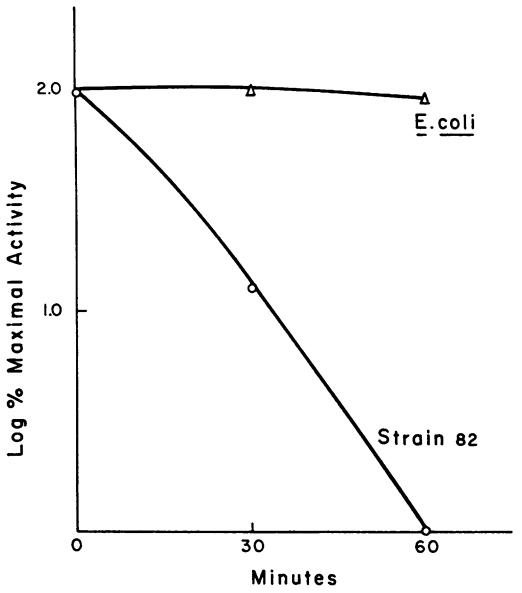

FIG. 7. *Heat inactivation at 46 C of cytochrome c reductase of strain 82 and Escherichia coli. The complete system consisted of 0.3 ml of 0.2 M Tris buffer (pH 7.5), 0.1 ml of 0.006 M $NADH_2$, 0.1 ml of 0.0012 M 2,6-dichlorophenol-indophenol, 0.1 ml of 0.03 M KCN, 0.04 mg of cell protein (0.01 ml), and 2.39 ml of water. The reaction was started by adding the protein. The final volume was 3.0 ml. The blank cuvette received all components except protein. Decreases in absorbance at 600 mμ were determined, and specific activities were calculated as decrease in absorbance per minute per milligram of protein at room temperature. There was no change in absorbance in the blank cuvette.*

TABLE 3. *Effect of heating at various temperatures on cytochrome c reductase of strain 82 and Escherichia coli*

Temp of heating	Specific activity[a]	
	Strain 82	*E. coli*
C		
Unheated	2.59	3.86
37	2.90	4.03
40	0.75	4.10
45	0.00	3.13

[a] Absorbance per minute per milligram of protein at 600 mμ. See Fig. 7 for assay conditions.

DISCUSSION

Evidence is accumulating which indicates that enzymes and enzyme synthesis in psychrophilic microorganisms are unusually heat-labile. Psychrophilic *Pseudomonas fragi* does not form lipase above 20 C (17). Inability of a psychrophilic *Cryptococcus* strain to grow above 30 C was shown by Hagen and Rose (10) to be due, probably, to inactivation of aconitase and fumarase. Burton and Morita (3) found that malic dehydrogenase in a psychrophilic marine vibrio is inactivated at 30 C, the maximal growth temperature of the bacterium. Also, the fermentation of glucose by psychrophilic *Candida* sp. P16 was completely abolished at 35 C, whereas that of mesophilic *Saccharomyces cerevesiae* was maximal at this temperature (18). In a recent investigation, Evison and Rose (6) found that, when exponentially growing cells of psychrophilic strains of *Arthrobacter*, *Corynebacterium*, and *Candida* were heated at temperatures 3 to 5 C above their maximal growth temperatures, respiratory activity decreased, and they correlated this with a decrease in the activities of the tricarboxylic acid cycle enzymes.

In previous investigations of psychrophile strain 82, it has been shown that formic hydrogenlyase and hydrogenase are not synthesized by strain 82 above 20 C but are formed by *E. coli* and other mesophilic bacteria at 45 C. Also, once formed, formic hydrogenlyase of strain 82 is completely inactivated at 45 C, compared to 70 C for that of *E. coli*. Psychrophile hydrogenase also is more heat-sensitive than is mesophile hydrogenase (21, 22). Furthermore, in the present investigations, the oxidative and fermentative metabolism of strain 82 were completely abolished by exposure of the cells to 46 C for about 1 hr, whereas those of *E. coli* were unaffected. More specifically, $NADH_2$ oxidase and cytochrome *c* reductase in strain 82 were rapidly inactivated at 46 C.

Not all of these heat-labile enzymes and enzyme-forming systems in the psychrophilic microorganisms are essential for growth. *P. fragi* can grow without lipase, and strain 82 can grow without formic hydrogenlyase and hydrogenase. However, the temperature-sensitive tricarboxylic acid cycle enzymes of *Cryptococcus*, *Candida*, *Arthrobacter*, and *Corynebacterium* species, the malic dehydrogenase of the marine vibrio, and the $NADH_2$ oxidase and cytochrome *c* reductase of strain 82 appear to be essential, at least for aerobic growth.

It is possible that the unusual heat sensitivity of enzymes and enzyme synthesis in psychrophiles may be correlated with the ability of these organisms to grow at low temperatures, in that these same heat-sensitive enzymes may function better at low temperatures than the corresponding relatively heat-stable enzymes of mesophiles. Comparative experiments with purified enzymes of psychrophiles and mesophiles are needed to explore this possibility.

ACKNOWLEDGMENT

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