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G.R. Chaudhry
Oakland University
Rochester, Michigan

A. Rashid Bhatti
Defence Research Establishment Suffield

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High Temperature Stress Induced Changes in *Pseudomonas aeruginosa*: Enzymology

by

A. Rashid Bhatti[1] and G.R. Chaudhry[2]

[1]Medical Countermeasure Section, Defence Research Establishment Suffield, Ralston, Alberta
[2]Department of Biological Sciences, Oakland University, Rochester, Michigan, USA

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ABSTRACT

Growth of *Pseudomonas aeruginosa* at 46°C is accompanied by an almost complete inhibition of periplasm-located alkaline phosphatase (APase) and cyclic-21,3'-phosphodiesterase, whereas 5'nucleotidase and ribonuclease I (RNase 1) are only partially affected. Cytoplasmic membrane enzymes such as glucose and gluconate dehydrogenases and cytochromes a, b and c are partially affected by growth at 46°C. When a culture grown at 37°C is shifted to 46°C, the cytochrome levels are decreased indicating that the high temperature inactivates the respective proteins. Growth of *P. aeruginosa* at 46°C had virtually no effect on the cytoplasm-located enzymes such as glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase which were examined. The apparent rates of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein synthesis of 46°C grown cells are similar to those of 37°C cells, however, the data indicate that transport systems may be impaired in 46°C cells. The synthesis of APase is not inhibited in 46°C grown cells and experiments performed in the presence of chloramphenicol and trypsin suggest that APase is secreted to the periplasm as a heat labile monomer which is unable to dimerize to form active enzyme.

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INTRODUCTION

Microorganism in particular bacterial species behave differently to survive under a variety of stresses, which results in the changes in the morphology as well certain biological functions of the respective organism (1, 2, 4, 17,18). *Pseudomonas aeruginosa* is a mesophilic, Gram-negative, bacterium which synthesizes a repressible alkaline phosphatase (EC 3.1.3.1.; APase) under inorganic phosphate (Pi) limiting conditions (6). The enzyme under normal growth conditions at 37°C is excreted to the growth medium, localized in the periplasm or on the outer surface of the outer cell wall (7). Culture of the organism under similar growth conditions at 46°C results in production of filaments (2). The filaments contain discrete chromosomal packets, the number of which does not exceed eight. This cell form contains little or no APase and the filament is induced to fragment after the addition of purified APase or Pi or by a temperature shift to 37°C (2). The data suggested that under these growth conditions APase plays a role in the cell division process.

The activity of the respiratory system is also altered after growth at 46°C such that the levels of the two oxidases o and a_1 were reduced eight- and six-fold respectively, whereas, cytochromes b and c were reduced to a lesser extent (5). Respiration studies indicated that growth at the elevated temperature resulted in reduced oxidation rates of certain substrates. The substrates most drastically affected appeared to require the participation of periplasm or membrane-located proteins. All of the aforementioned effects were reversible after a temperature shift to 37°C.

The studies just described suggested that some cellular processes, such as division, were severely affected after culture at 46°C whereas other phases such as: growth, *i.e.*, elongation of the outer cell wall and the cytoplasmic membrane; the synthesis of certain proteins, and chromosomal replication were only partially affected. In addition, a more detailed examination of various enzymes and cellular processes was undertaken and evidence is presented to suggest that periplasm-located proteins are most severely affected by high-growth temperatures whereas cytoplasmic membrane proteins are affected partially and cytoplasm-located enzyme activities remain relatively unaffected.

MATERIALS and METHODS

Organism and culture conditions: *Pseudomonas aeruginosa* (ATCC 9027) was grown aerobically in a Pi deficient medium (6) at 37 and 46°C as described elsewhere (2). Cells were washed in 15 mM tris (hydroxymethyl) aminomethane (Tris) HCl buffer (pH 7.0) and resuspended at approximately 10 to 20 mg protein/ml. Cell-free extracts were obtained by ultrasonic disruption (4 x 15 sec) at maximum output using an M.S.E. (model NT-20N) or a cell disruptor (model W185D) Branson Sonic Power Company, Plainview, New York. The disrupted cells were centrifuged at 36,000 x g for 10 min and the supernatant fluids were assayed for the respective enzymes.

Chemicals and Reagents: Para-nitrophenol phosphate (pNPP), Tris, bovine serum albumin (BSA), trypsin, chloramphenicol (CM), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), D-glucose-6-phosphate, DL-glyceraldehyde-3-phosphate (diethylacetal, monobarium salt), DL-isocitrate, were obtained from the Sigma Chemical Co., St. Louis, Mo. Imidazole was obtained from Eastman Organic Chemicals, Rochester, N.Y. Uracil-2^{14}C (56.82 mCi/mM) was obtained from New England Nuclear Corp., Boston, Mass. All other chemicals were of reagent grade obtained from Fisher Scientific Co., Montreal, Canada; glass distilled water was used throughout.

Estimation of protein: The method of Lowry *et al* (13) was used to estimate protein concentrations with bovine serum albumin as a standard.

Enzyme assays: All assays were carried out spectrophotometrically with the aid of a Gilford model 300-N spectrophotometer. APase, 5'-nucleotidase, ribomiclease, cyclic-2',3'-phosphodiesterase, isocitrate dehydrogenase, glyceraldehyde-3 -phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, glucose and gluconate dehydrogenase activities and cytochrome concentrations were assayed according to published procedures (6,16,9,21,14,20). The glucose-6-phosphate dehydrogenase assay was similar to that described by Malamy and Horecker (14) except that 0.05 M imidazole buffer, pH 8.0, was used in place of Tris buffer, pH 7.65. DL-glyceraldehyde-3-phosphate diethylacetal, monobarium salt was hydrolyzed (15) and adjusted to pH 7.5 with 0. 1 N NaOH. One unit of enzyme activity in all cases represents the conversion of 1 umole of substrate to product per min, and specific activity is units/mg of protein.

DNA and RNA synthesis: DNA and RNA synthesis were followed at 37 and 46°C after simultaneous incorporation of uracil-2-^{14} C according to the method of Deutch and Pauling (10). *P. aeruginosa,* grown to mid-log phase, was harvested and resuspended into the same volume of fresh medium and incubated at 37 and 46°C. After 10 min of equilibration at the respective temperatures labelled uracil was added to each flask. Portions (0. 1 ml) of the bacterial suspension were removed from cultures at the respective temperatures and one was added to 5 ml of ice cold trichloroacetic acid (TCA) (5% w/v) and the other was added to 0.5 ml of 2 N KOH at 37°C. After incubation for 2.5 h at 3 7°C, 4.5 ml of cold TCA (5 % w/v) was added to each KOH sample. Samples were filtered and washed with TCA (10% w/v). Alkaline sensitive, acid precipitable radioactivity (RNA) was calculated by subtraction of the alkaline resistant, acid precipitable radioactivity (DNA) from the total acid-precipitable radioactivity.

RESULTS

Effect of growth temperature and shift on periplasm-, peripheral membrane and cytoplasm-located enzymes: Crude extracts of cells grown at 37, 46 or 46°C and shifted to 37°C were examined for representative enzyme activities which are located in the periplasm, cytoplasmic membrane and the soluble cytoplasmic fractions (Table 1). Enzyme activities which

are located in the periplasm (3) such as APase, 5'-nucleotidase, cyclic-2', 3'-phosphodiesterase and nibonuclease 1 (RNase) are completely or partially eliminated by growth at 46°C. RNase 1 appears to be an exception but a reduction in activity is always observed at 46°C. When the 46°C grown cultures are shifted to 37°C, the activities of the periplasm-located enzymes returned to the normal 37°C level. Peripheral membrane-located enzymes such as glucose, or gluconate dehydrogenases, are only partially affected *(i.e.,* 60% reduction) after growth at 46°C and the levels returned to normal following a shift to 37°C. Enzymes representative of a cytoplasmic location such as; glucose-6-phosphate, isocitrate and glyceraldehyde-3-phosphate dehydrogenases are not affected by growth at 46°C and the levels may be higher after growth at 46°C or after a shift to 37°C.

The results of the experiments just described were obtained by assaying crude extracts of cells grown for 12 h under the described conditions. Since changes in enzyme activities could result at any time during the growth cycle, the experiments were repeated and specific activities were examined at various times (Table 2). In this experiment cytochromes a, b and c were selected as cytoplasmic membrane-located activities. When a culture is initially grown at 37°C and shifted to 46°C slight increases in the activity of periplasm-located enzymes such as APase and 5'-nucleotidase are observed. This experiment also shows that preformed, periplasm-located enzymes are not inactivated or destroyed by a shift to 46°C growth. The levels of cytochromes are affected by a shift to 46°C and there is a decrease in over-all concentrations during the 3 h at 46°C. As reported in the previous experiment, there is no effect on the appearance of representative cytoplasm-located activities. When cultures are grown initially at 46°C, however, there is virtually no synthesis of the periplasm-located enzymes whereas the levels of cytochromes are differentially affected, *i.e.,* cytochromes a and b are initially similar to the levels obtained in 37°C grown cells but c is reduced to approximately 45%. During growth at 46°C there is a constant decrease in the specific activity levels of these proteins and the levels are returned to normal after a shift to 37°C. Cytoplasm-located activities are not affected by growth at 46°C and the specific activities continue to increase. In addition, the activities continue to increase after a shift to 37°C.

High temperature effects on macromolecular reactions: Although cells of *P. aeruginosa* do not divide at 46°C, they do form filaments and consequently outer wall and membrane components must be synthesized and inserted into the elongating cell. In addition, it is apparent that the mass of the filament must increase and consequently DNA, RNA and protein must be synthesized to contribute to the mass. Results obtained in a previous section showed that most periplasm-located proteins examined were severely or completely inhibited by growth at 46°C. The most drastic observed effect was upon APase and this result was unexpected since the enzyme normally is stable to heat (2). Temperature shift experiments from 46 to 37°C reported in a previous study (2) and in this communication indicated that the appearance of APase is almost immediate after the shift suggesting that perhaps APase, as well as other periplasm-located proteins, may be present as catalytically inactive species. The results of a typical experiment are reported in Fig. 1. The addition of CM to a culture growing at 37°C caused considerable cell disintegration due to lysis (Fig. 1A) and debris was observed by phase

microscopy. When CM was added to cultures growing at 46°C before shifting to 37°C, a slight increase in A_{660} nm occurred, there was no observable lysis and cells were motile, dividing and single or in pairs. APase in the culture filtrate of 37°C grown cells increased slightly after the addition of CM (Fig. 1B) then decreased rapidly due to the decrease in pH of the filtrate (pH 6.7 to pH 5.05). Although the quantity of APase in the culture filtrate of 46°C grown cells is initially small, there is a rapid increase in the enzyme level after a shift to 37°C and it attains or surpasses the level normally obtained in 37°C grown cells (3). However, if the same experiment is repeated in the presence of CM (Fig. 1B) only a 30% increase in activity is noted after the shift to 37°C. If the experiment is repeated but 10 ug/ml of trypsin is included with the CM there is no additional appearance of APase above the control level. These experiments indicate that APase which is preformed at 37°C is stable to both CM and trypsin treatment.

The data of Fig. 2A show that the rate of incorporation of uracil-2-^{14}C into RNA increased during the first 40 min after incubation of 46°C grown cells. At 46°C (curve 2) this rate is slower when compared to the incorporation of uracil into the RNA of cells grown at 37°C (curve 1). Incorporation of uracil ceases from the 40th to the 60th min in cultures incubated at 46°C and resumes when the cultures are shifted to 37°C (curve 2). The rate of incorporation of uracil into RNA increased in cells at 37°C linearly up to 30 min and after this time decreased. A temperature shift of this culture to 46°C did not have a detrimental effect upon incorporation. When the concentration of uracil (0.0321 mM) was increased 10-fold and the experiment repeated it was found that the rate of incorporation of uracil at 46°C was the same as that at 37°C and there was no effect on incorporation of uracil after a shift of 46°C cells to 37°C (curve 3) as observed in curve 2.

The incorporation of labeled uracil into DNA gave results similar to those obtained for RNA. Incorporation of uracil into DNA was linear for up to 30 min in 37°C grown cultures and no effect was observed after a shift to 46°C (curve 1). Incorporation of uracil into the DNA of 46°C cells was initially slow but increased after a shift to 37°C (curve 2). When the concentration of uracil was increased 10-fold, the rate of incorporation of uracil into 46°C cells increased and was linear for up to 20 min (curve 3). The initial rate of incorporation of uracil was similar to that obtained with 37°C grown cells. When the 46°C culture was shifted to 37°C and these cells assayed, there was an increase in the rate of incorporation of uracil and this rate was similar to the initial rate observed with 37°C grown cells. Similar experiments were also performed to test the effect of growth temperature on the rate of protein synthesis. Using the stable incorporation of leucine-2-^{14}C into protein, results similar to those reported for RNA and DNA synthesis were obtained (data not shown). As determined above, the apparent decreased rate of incorporation of leucine at 46°C could be off set by increasing the leucine concentration.

The foregoing data indicate that perhaps the transport systems responsible for uracil or leucine uptake may function more slowly or are partially inhibited after growth at 46°C. To test this hypothesis the kinetics of uracil incorporation were studied with 37 and 46°C grown cells and typical results are presented in Fig.3. Although the Vmax of uracil incorporation is decreased 40% in 46°C cells, it is more pertinent to note that the Km for uracil is increased by

approximately 3-fold (3.33 x 10^{-6} M for 37°C as compared to 1.05-x 10^{-5} M for 46°C cells). These alterations in Km and Vmax may explain why the rate of uracil incorporation into the 46°C grown cells may be increased simply by increasing the uracil concentration.

DISCUSSION

Temperature is an environmental factor that can modulate gene expression (17) and is well established that expression of bacterial virulence determinants is modulated by changes in environmental conditions (22). *P. aeruginosa* forms long, undivided, non-motile filaments at high temperature (2) and become sensitive to wide variety of antibiotics (4). Under these conditions cells get larger and there is net synthesis of some representative enzymes whereas other enzymes, or protein systems decrease over the time period examined. It is evident from the results that the initial culture used for inoculation is unable to divide, *i.e.,* filaments are formed. It is also evident that there is a net increase in some cellular components such as the outer cell wall, mucopeptide and the cytoplasmic membrane as well as a net increase in internal enzymes. Conditions for growth at 46°C, therefore, may be divided into at least three stages, some of which are more affected than others. The stages consist of 1) division, 2) growth and duplication of essential components such as proteins which result in a mass increase and which require energy and, finally, 3) replication of critical information such as DNA and RNA. The only stage which appears to be completely blocked at 46°C is division.

Detailed examination of enzymes and systems which are located in various areas of the cells suggest that periplasm-located enzymes (12) are the most severely affected following growth at elevated temperatures. Enzymes such as APase, 5'-nucleotidase and cyclic2',3'-phosphodiesterase are completely or almost completely absent in cells grown in 46°C. However, the shift to 37°C results in an immediate resumption in the appearance of these enzymes. APase was further examined as a model and experiments performed in the presence of CM and the proteolytic enzyme trypsin, following a shift from 46 to 37°C, suggest that the normally dimenic APase appears as a monomer at 46°C since only this species is subject to proteolysis by trypsin (8). It was also demonstrated that monomer units are synthesised and secreted at 46°C since a shift from 46 -37°C in the presence of CM alone results in the appearance of some APase activity. In addition, the filamentous cells also become motile following a temperature shift and it is concluded that preformed flaggelin monomers are able to aggregate under conditions which exist after the shift. RNase 1 appears to be an exception to the observations and its activity is only slightly reduced at 46°C. This may be due to the fact that this enzyme is normally a monomer in its active form. It is concluded that high growth temperature is a competing force with polymerization of protein monomer units and that generally the monomers are more heat labile as compared to the polymeric, active forms of the proteins. This conclusion is further strengthened by the fact that preformed periplasm-located enzymes at 37°C are generally stable following a shift to 46°C.

Representative peripheral cytoplasmic membrane-located enzymes such as glucose and gluconate dehydrogenases are partially affected by growth at 46°C as are the levels of cytoplasmic membrane activities such as the cytochromes. However, none of the activities examined are completely eliminated as determined for periplasm-located activities. The most interesting finding is the fact that none of the cytoplasm located activities examined are effected by growth at 46°C. In some cases, specific activities of the enzymes increased at the elevated temperature.

Intracytoplasmic macromolecular synthetic reactions such as those of DNA, RNA and protein are relatively unaffected in 46°C grown cells. Evidence for this statement is supported by the observation that under the proper assay conditions the rates observed in 46°C grown cells are the same as those observed in 37°C cells. It is apparent, therefore, that these reactions are not rate limiting in regard to cell division. Kinetic experiments indicate that the rate limiting step may be the uptake or transport of the particular compound, i.e., uracil or leucine, under study. The increased Km and the decreased Vmax values suggest that the decreased rates of macromolecule synthesis observed in 46°C grown cells may be due to reversible conformational alterations which occur in one or more proteins of the transport system. Since these components are usually associated with a periplasm or peripheral membrane location, the results of the previous section of this study strengthen this view rather than the alternative view concerning a direct effect upon the enzymes involved specifically with the synthesis of the macromolecule.

Cells of *P. aeruginosa* grown at 46°C appear to possess all the necessary requirements for increased cell size and mass and for the duplication of cytoplasmic components. The only readily apparent difference of 46°C grown cultures is their inability to divide. Although the energy generating capacity of these cells is reduced, the stoichiometries observed suggest that they are complete and conserved. The most drastic effect of high growth temperatures appears to be localized to enzymes and metabolic functions of the periplasm with intermediate effects upon functions of the cytoplasmic membrane, dependent upon the membrane face location and, finally, no apparent effect upon intracytoplasmic located functions. The gradation of effect by temperature as one moves towards a central cell location cannot be explained precisely at the moment although it might relate to the ordered state of water in the cell's cytoplasm which could protect enzymes from heat inactivation (12). Preliminary evidence has recently been obtained which shows that the intracellular concentration of potassium of 46°C grown cells is 2-fold greater than that of 37°C grown cells whereas the intracellular sodium concentration is identical. Potassium, in the present context, may play a role similar to that of calcium in thermophilic bacteria (19) or spores (11).

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TABLE 1. Variation in enzyme levels at different growth temperatures.

Enzyme	Activity (units/10ml)			Specific Activity (units/mg protein)		
Periplasm	37°C	46°C	46°C→37°C	37°C	46°C	46°C→37°C
Alkaline phosphatase	10.3	0.00	8.630	0.12	0.00	0.12
5-Nucleotidase	26.7	1.080	21.000	0.31	0 01	0.28
Cyclic-2',3'-phospho-diesterase[a]	0.9	0 000	0.750	0.01	0.00	0.01
Ribonuclease I	0.3	0.2	0.3	0 003	0.002	0.003
Cytoplasmic membrane						
Glucose dehydrogenase	10.2	0.4	9.8	0.12	0.05	0.13
Gluconate dehydrogenase	8.9	2.4	9.0	0 10	0.03	0.12
Soluble cytoplasmic						
Glucose-6-phosphate dehydrogenase	10.3	13.9	11.3	0.12	0.18	0.15
Isocitrate dehydrogenase	22.4	23.9	21.8	0.26	0.31	0.29
Glyceraldehyde-3-phosphate dehydrogenase	3.4	3.9	3.8	0.04	0.05	0.05

Note: Cells were grown at 37 or 46°C for 12 h after inoculation into P_i limiting media At the end of this time representative samples, (10 ml) were removed, centrifuged and the cell pellets were resuspended into Tris buffer and ultrasonically disrupted. The remaining culture, initially grown at 46°C, was transferred to 37°C and after 3 h at this temperature the cells were assayed for the respective enzyme activities. The data reported are for one complete experiment. Similar results were obtained in other trials.

[a] Since this enzyme appears in the particulate fraction, the crude extract was assayed before centrifugation.

TABLE 2. Effect of temperature shift on the enzyme activities of *P. aeruginosa*.

Activity Measured	Specific Activity at time t X 10^{-2}											
	2		4		6		7		9		11	
Alkaline phosphatase	1 2	2 2	1 3	2 1	1 10	2 1	1 12	2 5	1 13	2 8	1 -	2 12
5'-nucleotidase	3	3	4	-	5	1	5	5	5	6	-	9
Cytochrome												
a	6	6	7	5	7	5	6	7	4	7	-	7
b	12	11	22	11	22	9	15	15	13	17	-	17
c	25	14	35	14	34	9	22	17	21	22	-	21
Glucose-6-phosphate dehydrogenase	7	8	13	10	13	18	13	17	14	23	-	24
Isocitrate dehydrogenase	4	3	9	2	10	14	10	17	11	2	-	24
Glyceraldehyde-3-phosphate dehydrogenase	2	1	3	3	5	6	4	6	6	6	-	11

Note: Cultures were grown at an initial temperature of 37°C (column 1) or 46°C (column 2) for 6 h. After this time the cultures were shifted to 46 or 37°C respectively. Ten ml samples were removed at the indicated time and enzyme activity determined as described in Table 1. Enzyme activities are expressed as μmoles/min/mg protein while cytochromes are expressed as nmoles/mg protein.

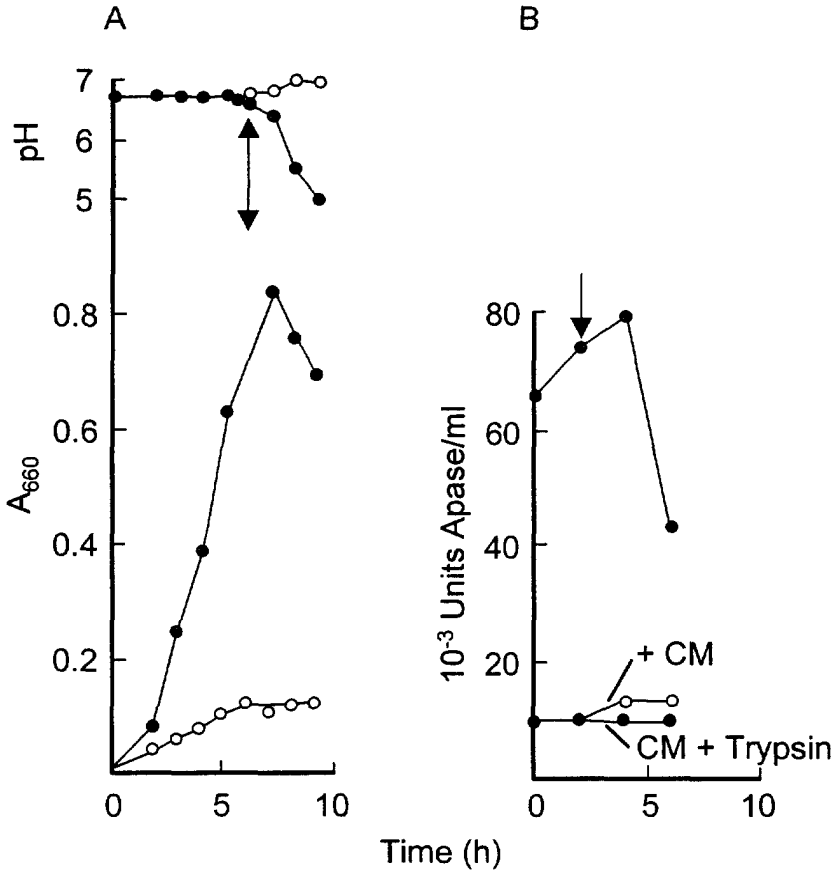

Figure 1. The effect of chloramphenicol and trypsin on APase of P *aeruginosa*. The cultures were grown at 37 and 46°C. After 6 h of growth CM (400 ug/ml) was added to both cultures. Cultures grown at 46°C were aseptically divided into two equal portions. Trypsin (10 ug/ml) was added to one portion and none to the other. The 3 flasks were incubated at 37°C. At the indicated time intervals, samples were withdrawn and analyzed for pH, A_{660} nm and APase activity.

A. Growth and pH changes at 37 and 46°C. An arrow indicates the time of addition of CM to the cultures and the temperature shift to 37°C for cultures grown at 46°C. Solid and hollow circles represent A_{660} nm and pH at 37 and 46°C, respectively.

B. The upper curve (solid circles) represents APase activity in the absence and presence of CM at 37°C. The arrow indicates the time when CM was added. In the lower curve hollow and solid circles represent APase activities in the presence of CM and CM + trypsin, respectively. The arrow also indicates the temperature shift from 46 to 37°C.

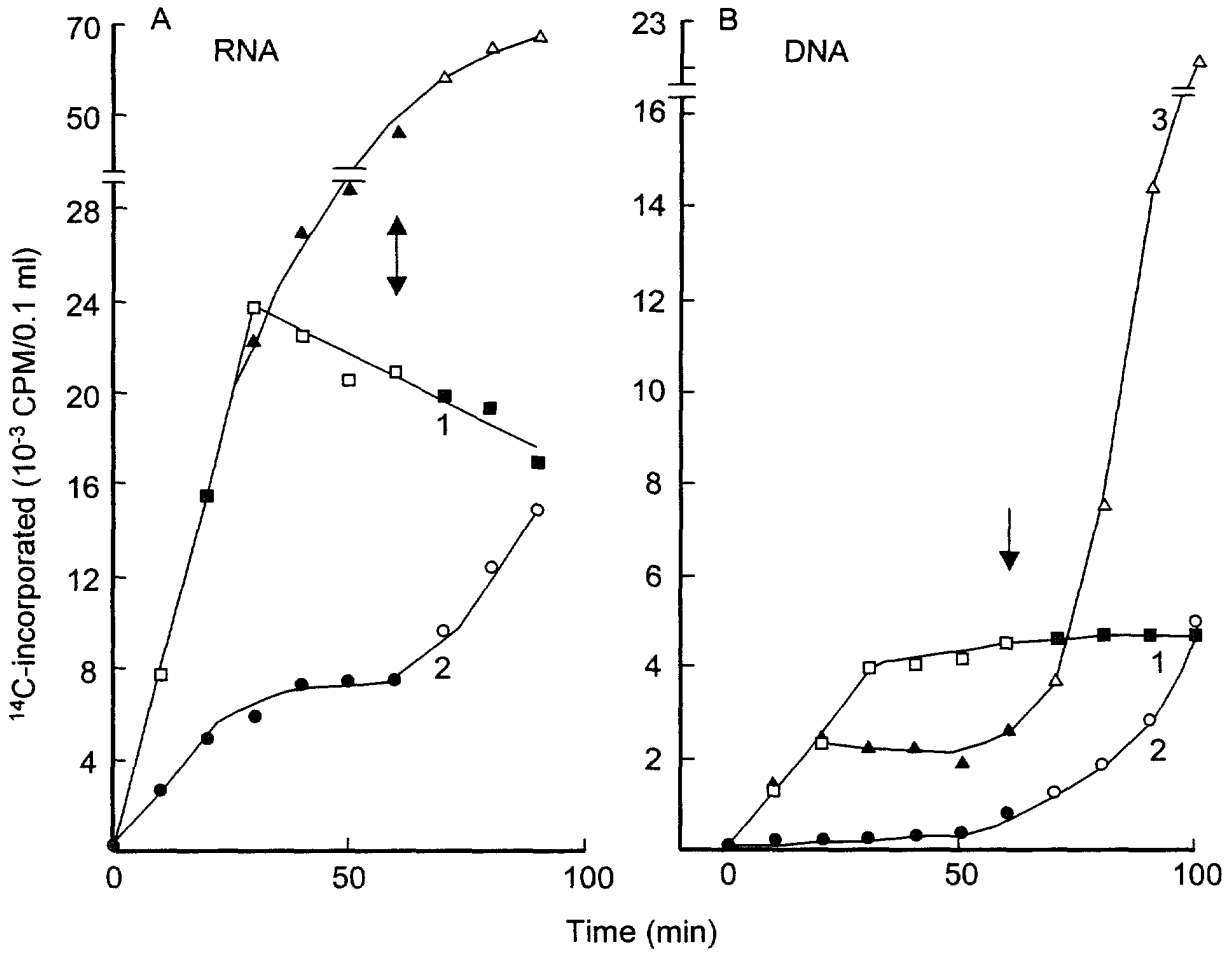

Figure 2. Effect of temperature shift on RNA and DNA synthesis at 37 and 46°C. The culture was grown at 37 and 46°C to 0.45 absorbance units. Thirty ml of culture was centrifuged and the cells were resuspended into the same volume of fresh medium and divided equally into three 50 ml flasks. One flask was incubated at 37°C and the other two incubated at 46°C. After 10 min of equilibration at the respective temperatures, 10 ul of uracil-2-^{14}C was added to flask I at 37°C, 10 ul and 100 ul uracil-2-^{14}C were added to flasks 2 and, 3 at 46°C. At the indicated time intervals samples were removed and analysed for RNA (A) and DNA (B) as described in Methods and Materials. The temperature shift is indicated by an arrow. Symbols: Hollow and solid cubes and circles represent the culture containing 10 ul of uracil at 37 and 46°C respectively. Hollow and solid triangles represent cultures containing 100 ul of uracil at 46 and 37°C.

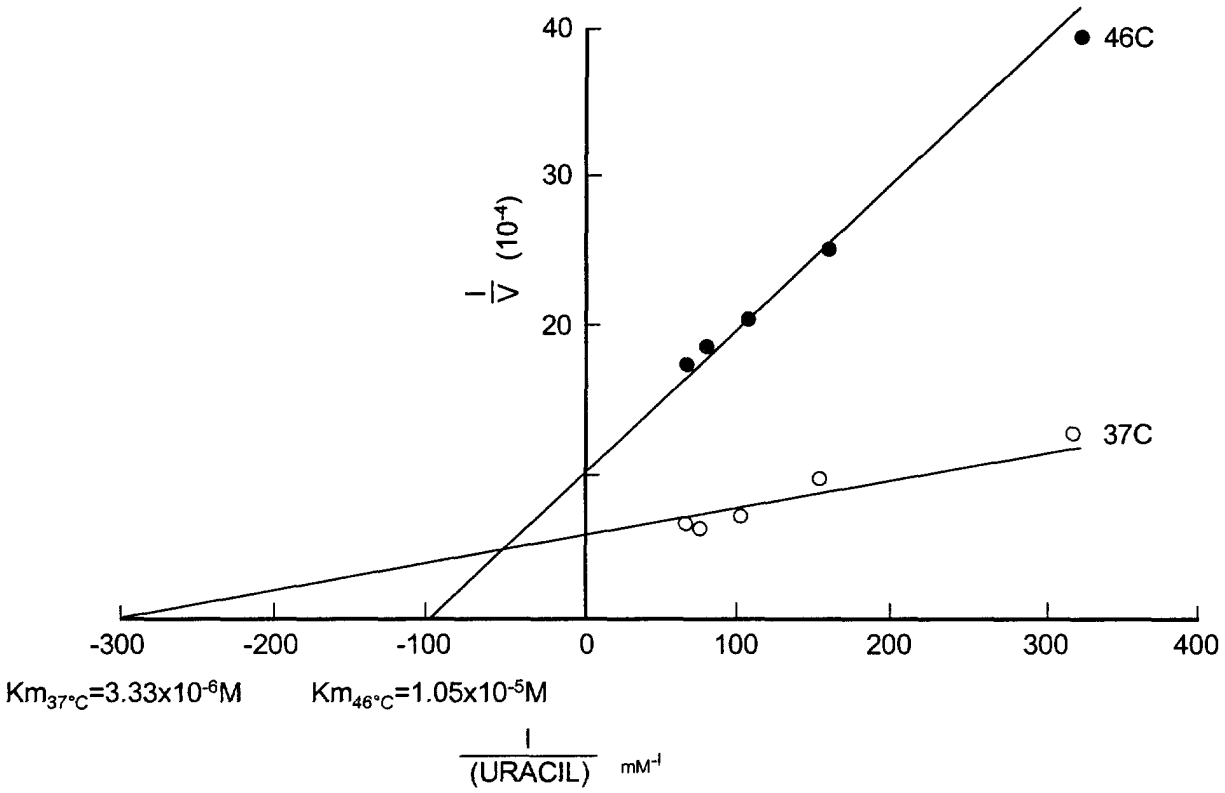

Figure 3. Kinetics of uracil-2-^{14}C incorporation into nucleic acid at 37 and 46°C. Growth of the culture is described in the legend to Fig. 3 The rate of incorporation of uracil-2-^{14}C into nucleic acid was calculated per mg of protein.

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Growth of *Pseudomonas aeruginosa* at 46°C is accompanied by an almost complete inhibition of periplasm-located alkaline phosphatase (APase) and cyclic-21,3'-phosphodiesterase, whereas 5'-nucleotidase and ribonuclease 1 (RNase 1) are only partially affected. Cytoplasmic membrane enzymes such as glucose and gluconate dehydrogenases and cytochromes a, b and c are partially affected by growth at 46°C. When a culture grown at 37°C is shifted to 46°C, the cytochrome levels are decreased indicating that the high temperature inactivates the respective proteins. Growth of *P aeruginosa* at 46°C had virtually no effect on the cytoplasm-located enzymes such as glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase which were examined. The apparent rates of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein synthesis of 46°C grown cells are similar to those of 37°C cells, however, the data indicate that transport systems may be impaired in 46°C cells. The synthesis of APase is not inhibited in 46°C grown cells and experiments performed in the presence of chloramphenicol and trypsin suggest that APase is secreted to the periplasm as a heat labile monomer which is unable to dimerize to form active enzyme.

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P. aeruginosa

Enzyme

Periplasm

Cytoplasm

Cytochrome

Temperature

Nucleic acid

Protein

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