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*Thiobacillus concretivorus*의 代謝經路에 關한 酵素學的 硏究

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The Enzymatic Studies on Metabolic Pathways in *Thiobacillus concretivorus*

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ABSTRACT

A study was made on enzymes of carbohydrate metabolism in *T. concretivorus* grown with and without glucose. The present results show that *T. concretivorus* possesses high activities of pentose shunt pathway and related enzymes, glucokinase, G-6-P dehydrogenase, 6-PG dehydrogenase, and phosphoglucoisomerase, but low activities of enzymes unique to EMP(fructose-1,6-diphosphate aldolase). Although the synthesis of the latter enzymes remains largely unaffected by the growth environment, that of the former is stimulated by glucose. And the failure to detect ED pathway enzymes in cells grown in thiosulfate or thiosulfate-glucose medium eliminates the ED pathway as a significant route of glucose catabolism in *T. concretivorus*. These results suggest that pentose shunt pathway performs an energetic role in glucose metabolism by *T. concretivorus* with EMP as a subway. The absence of ED pathway and the presence of pentose shunt pathway which is the major route of catabolism in *T. concretivorus* are similar to those of other obligately chemolithotrophic thiobacilli.

The G-6-P and 6-PG dehydrogenase are both NAD and NADP specific, but NAD predominant. However, the 3-PGAL dehydrogenase is only NAD specific. Since the specific activity of 3-PGAL dehydrogenase in thiosulfate-glucose grown cells is high, it is possible that 3-PGAL generated from glucose is converted mainly into pyruvate which is channeled into the TCA cycle.

All enzymes of the TCA cycle tested and NADH oxidase are detected in the cells of *T. concretivorus* grown in thiosulfate. The specific activities of fumarase and isocitrate dehydrogenase are high and others are low. The presence of two isocitrate dehydrogenase (NAD- and NADP- linked) may have important regulatory function for this organism.

The activity of NAD-oxidase, which is implicated in the energy generating metabolism, was very high in the crude cell-free extract of *T. concretivorus*, recording 55.11 mμ mole/min/mg protein. This well coincides with the fact that activities of NAD-linked G-6-P dehydrogenase, 6-PG dehydrogenase and 3-PGAL dehydrogenase were high.

INTRODUCTION

Members of the genus *Thiobacillus*, generally called the "Nonphotosynthetic sulfur bacteria", are gram negative, nonsporulating rods measuring 0.5 by 1—3 μ, which, except for the nonmotile *T. novellus*, are polar flagellated. All members of the genus are known to oxidize reduced sulfur compounds such as thiosulfate *via* various pathways and use carbon dioxide as carbon source.

This great versatility makes it difficult to map a general metabolic pathway for these organisms. The four general pathways recognized at the present moment by Parker and Prisk (1953), and Parker(1957) are the followings:(1) Here, thiosulfate is oxidized first to the tetrathionate, which in turn is reoxidized to sulfate. However, the organisms which apparently perform this reaction are not able to oxidize tetrathionate alone; *T. concretivorus*, *T. thiooxidans* and *T. neapolitanus* are the only bacteria in this group which oxidize tetrathionate. (2) *T. thioparus* oxidizes thiosulfate first to sulfur, which in turn is oxidized to sulfate. (3) The facultative autotroph *T. novellus* is able to oxidize thiosulfate directly to sulfate (Aleem, 1965). (4) Some facultative autotrophs are able to perform this reaction which is coupled with a rise in *p*H that is sometimes followed by a return to the original value. Although some of the thiobacilli are able to form elemental sulfur, they never seem to store it, but rather oxidize it further or excrete it.

Obligate chemolithotrophic bacteria are characterized by their ability to grow with a specific inorganic nutrient as a source of energy, their ability to obtain all of their cell carbon from CO_2, and their inability to grow on organic nutrients in the absence of adequate quantities of their inorganic energy source. There have been four explanations for the latter property. Winogradsky (1890), Borichewski (1967) first proposed that organic matter was toxic to them, but, clearly, certain organic materials are not toxic (Hah and Ju, 1972). Glucose, for example, can be added to cultures containing adequate specific inorganic energy sources without discernible inhibition or stimulation (Vishniac, 1949; Borichewski and Umbreit, 1966; Matin, 1969; Matin and Rittenberg, 1970 and 1971; Smith and Hoare, 1968). It was then postulated (Oginsky and Umbreit, 1959) that the obligate chemolithotrophic bacteria were "the physiological examples of the submarines" in that they had walled off an external toxic environment, permitting only their specific inorganic energy sources, CO_2, and a few mineral salts to enter. When radioisotope tracers became available, it was shown that this supposition was incorrect (Smith and Hoare, 1968). A wide variety of organic materials was found to penetrate such cells and be utilized (Lundgren, Andersen, Remsen and Mahoney, 1964; Shafia and Wilkinson, 1969). For example, *T. thiooxidans* in the presence of sulfur and CO_2 could absorb aspartic acid and convert it into cellular protein, other amino acids, ammonia, and CO_2. However, aspartic acid could not serve as a source of energy or nitrogen, and it could not replace CO_2 (Butler and Umbreit, 1966). Recently, Smith, London, and Stanier(19

67) and Hooper(1969) surveyed a wide variety of chemolithotrophic and phototrophic organisms including blue-green algae and found that those incapable of growth on glucose in the absence of their specific energy source were lacking in at least two enzymes, α-ketoglutrate dehydrogenase and NADH oxidase. Other reports have shown that not all strains lack these enzymes(Butler and Umbreit, 1969; Hempfling and Vishniac, 1965; Skinner and Walker, 1961; Trudinger and Kelly, 1968). But clearly it is not necessary that all chemolithotrophic bacteria should lack the same enzymes, and the absence of one or more enzymes in these cells may be critical. A fourth hypothesis maintains that obligate chemolithotrophic bacteria will indeed grow on glucose if the conditions are properly adjusted and that, whatever enzymes may be lacking, this lack is not sufficient to prevent growth on glucose. The proper conditions for growth require that the medium be constantly renewed. It is supposed that glucose *per se* is not but that, when the cells are placed on glucose in the absence of their specific energy source, toxic materials are formed from glucose, whereas if the specific energy source is present these materials are not formed in the same quantity. This hypothesis is based upon observations showing that the acid-tolerant sulfur or iron bacteria could grow on glucose, at least for several transfers (Shafia and Wilkinson, 1969; Remsen and Lundgren, 1963), and that, if growth was conducted under conditions whereby the alleged toxic products were moved by dialysis, growth on glucose could be continued indefinitely (Borichewski and Umbreit, 1966). However, all of these latter studies were done with acid-tolerant bacteria capable of using sulfur as their specific energy source, and it is possible that these organisms are the only ones which were capable of growth on glucose under dialysis conditions(Pan and Umbreit, 1972).

Many investigators have been interested in examining the constitution of enzymes in mixotrophic, heterotrophic and autotrophic members of the genus *Thiobacillus*, to see which of the enzymes change when thiobacilli and other autotrophic organisms switch from an autotrophic existence to a heterotrophic one (Matin and Rittenberg, 1970 and 1971; Williams and Hcare, 1972; Tabita and Lundgren, 1971; Aleem, 1966; Hampton and Hanson, 1969; Johnson and Abraham, 1969; Trüper, 1965; Zegallow and Wang, 1967).

However, there are no enzymatic studies on carbohydrate metabolism in *T. concretivorus* which is obligate chemolithotroph. Accordingly, *T. concretivorus* was analyzed for key enzymes of the various pathways of carbohydrate metabolism.

EXPERIMENTALS

1) Organism and media.

Our laboratory strain of *T. concretivorus* was used in these studies(Hah, Cha, and Ahn, 1972). The mineral salts base used in the preparation of various media had the following composition in gram/1,000m*l* (w/v) : $(NH_4)_2SO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.01; KH_2PO_4, 4.0. This base was supplemented with $Na_2S_2O_3 \cdot 5H_2O$ or organic substrates and thiosulfate at

concentrations specified in previous papers.

Distilled, deionized water was used throughout. Phosphates, $FeSO_4$, $Na_2S_2O_3 \cdot 5H_2O$, and organic supplements were sterilized separately and added aseptically to the medium. Parallel stock cultures were maintained on the same media.

2) Growth procedures.

Growth experiments were done in 300 m*l* culture flasks containing 30—25m*l* of medium. The inocula used were grown in homologous media. Growth was followed by measuring increase in turbidity of cultures by using a Beckman DU-spectrophotometer with 550nm spectrum. A standard curve was prepared. Cultures were shaken on a reciprocal type shaker at 30°C. Incubation was continued until the stationary period was reached and growth yields were calculated from maximal turbidities. The *p*H levels of all of the culture were kept between 4.5 and 5.0.

Fernbach flasks containing 300 m*l* of medium were employed for obtaining large cell mass. Starter cultures on thiosulfate agar media were grown in Petri dish. Growth harvested from five to seven Petri dishes was used to inoculate a single flask. Otherwise, 100 m*l* of culture grown in the homologous medium was used per flask. The flasks were shaken at 30°C on a reciprocal shaker set at 120rev/min for seven days.

Contamination and purity was tested at the time of harvesting by microscopic examination and by streaking on the thiosulfate and thiosuflate-glucose agar plates.

Cells grown in 15 *l* media were harvested at 4°C in a International PR-2 centrifuge.

In order to obtain the physiologically active cells, cells harvested were resuspended in 500 m*l* of the thiosulfate and thiosulfate-glucose media and cultured at 30°C on a reciprocal shaker for 60 hours (late lag phase).

3) Preparation of cell-free extracts.

Cells grown for 60 hours were harvested at 4°C in a International PR-2 centrifuge, and suspended in 50mM tris-buffer(*p*H 7.9) or potassium phosphate (50mM)-glutathione(1mM) buffer(*p*H 7.4). Sulfur precipitate was then removed by centrifuging the suspension at 1,000 ×g for 20 min at 4°C. The cells contained in the supernatant fluid were collected by centrifugation at 15,000×g for 10 min.

The cells were washed three times with a buffer(*p*H 7.9) containing 50 mM tris-hydrochloride, 12 mM β-mercaptoethanol, 50 mM $NaHCO_3$, and 15 mM EDTA and then resuspended in this buffer overnight. This procedure is known to facilitate cell breakage of autotrophically grown thiobacilli; storage of whole cell at 4°C did not affect subsequent enzyme determinations (Silver and Lundgren, 1968). And then cells were frozen three times at the temperature of a dry ice and acetone mixture, thawed, and broken in water-cooled Biosonic III (20kc/sec) oscillator for 30 min.

Whole cells and debris were removed by centrifuging at 8,000×g for 10 min at 4°C. The crude supernatant fluid was then centrifuged at 25,000×g for 30 min at 4°C. The supernatant fluid was collected and used as crude cell-free extract for most of the enzyme assays.

TCA cycle enzymes were assayed by using cell-free extracts from cells pre-

viously harvested at 4°C by centrifugation and immediately suspended in a potassium phosphate(50mM)-glutathione(1mM) buffer at *p*H 7.4. The presence of glutathione in the buffer was essential for maximum enzymatic activity. The cells were suspended in aforementioned buffer and placed in the standard Raytheon cup, and N_2 gas was bubbled through the cell suspension for 10min. The cell was broken by an exposure for 30 min. Residual whole cells and debris were removed by centrifugation at 25,000×g for 30 min at 4°C. The supernatant fluid was separated and used for Krebs cycle enzyme assays as well as for NADH oxidase assays.

4) Enzyme assay.

The following enzyme assays were carried out spectrophotometrically by using standard technique(Colowick and Kaplan, 1966) with minor modifications; D-glucokinase [adenosine triphosphate (ATP): D-glucose-6-phosphotransferase, EC 2.7.1.2.]; glucose-6-phosphate dehydrogenase [D-G-6-P: nicotinamide adenine dinucleotide phosphate(NADP) and nicotinamide adenine dinucleotide (NAD) oxidoreductase, EC 1.1.1.49]; phosphogluconate dehydrogenase[6-phospho-D-gluconate: NADP oxidoreductase(decarboxylating), EC 1.1.1.44]; phosphogluconate dehydrase (phosphogluconate dehydratase, EC 4.2.1.12); 2-keto-3-deoxy-6-phosphogluconate(KDPG) aldolase, EC 4.1.2.14; glucosephosphate isomerase, (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9); phosphofructokinase(ATP: D-fructose-6-phosphate-phosphotransferase, EC 2.7.1.11); aldolase(fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13); 3-phosphoglyceraldehyde dehydrogenase[D-glyceraldehyde-3-phosphate: nicotinamide adenine dinucleotide (NAD) oxidoreductase (phosphorylating), EC 1.2.1.12]; succinate dehydrogenase[succinate (acceptor) oxidoreductase, EC 1.3.99.1]; Aconitase[citrate (isocitrate) hydrolyase, EC 4.2.1.2.]; isocitrate dehydrogenase[threo-DL-isocitrate: NAD(or NADP) oxidoreductase (decarboxylating), EC 1.1.1.41 and EC 1.1.1.42]; fumarase (fumarate hydratase, EC 4.2.1.2); α-ketoglutarate dehydrogenase [2-oxoglutarate: lipoate oxidoreductase (acceptor acylating), EC 1.2.4.2]; NADH oxidase [NADH: (acceptor) oxidoreductase, EC 1.6.99.3].

Hexokinase was measured spectrophotometrically by coupling its activity with endogeneous G-6-P dehydrogenase; the rate of NAD reduction was followed at 340nm, and the activity of the enzyme was calculated from the initial rate of NAD reduction. The reaction mixture (3m*l*) contained: 15mM tris-hydrochloride buffer(*p*H 7.9), 2μM $MgCl_2$, 2.5μM glutathione, 0.25μM NAD, 1.25μM ATP, 5μM hexose(glucose, fructose) and 1 m*l* of crude enzyme (Anderson and Kamel, 1966).

The activity of phosphoglucoisomerase was measured by the procedure of Cooper *et al.* (1958), which couples the production of F-6-P with G-6-P dehydrogenase. The reaction mixture contained 15mM tris-buffer(*p*H 7.9), 2μM $MgCl_2$, 2.5μM glutathione, 0.25μM NAD, 5μM F-6-P, and cell extract 1 m*l* and water to 3.0 m*l*.

Glucose-6-phosphate dehydrogenase assay mixtures (3.0m*l*) contained 15mM tris-hydrochloride buffer(*p*H 7.9), 6.67 mM $MgCl_2$, 0.83 mM D-glucose-6-phosphate(disodium salt), 0.33mM NADP or

NAD(*p*H 7.0), and cell free extract 1m*l*. The reaction was initiated by the addition of either NADP or NAD (Langdon, 1966). Under these conditions, the presence of 6-phosphogluconate dehydrogenase in the crude bacterial extracts did not interfere with determination of glucose-6-phosphate dehydrogenase. For example, the sum of glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities determined in separate reaction mixtures was equal to the total activity of these enzymes when both were measured simultaneously in the same reaction mixture (Lessie and Vander Wyk, 1972).

Reaction of 6-phosphogluconate dehydrogenase (3m*l*) were the same as for glucose-6-phosphate dehydrogenase except 2.67 mM 6-phosphogluconate was substituted for glucose-6-phosphate. The potassium salt of 6-phosphogluconate was prepared from the barium salt by the method of Horecker and Smyrniotis (1955). Fructose-1, 6-diphosphate aldolase was measured by using a coupled assay containing triose-phosphate isomerase and α-glycerophosphate dehydrogenase. The activity of the enzyme was calculated from the initial rate of NADH oxidation. The reaction mixtures (3.0m*l*) contained 0.1M tris-hydrcchloride (*p*H 7.5), 5mM fructose-1, 6-diphosphate, 0.25 mM NADH or NAD(*p*H 7.0), 0.02m*l* of α-glycerophosphate dehydrogenase-triosephosphate isomerase (10 mg/m*l*), and cell extract and water to 3.0m*l*. The reaction was initiated by adding cell extract (Taylor, 1955).

Phosphoglyceraldehyde dehydrogenase activity was measured as described by Hohorst *et al.*(1959). This is a coupled enzyme assay based on the formation of 1, 3-diphosphoglycerate which is then converted to 3-PGAL. The reaction mixture contained 0.1 M tris-buffer(*p*H 7.5), 10 μM $MgCl_2$, 8 μM reduced glutathione, 10 μM ATP, 10μM 3-phosphoglycerate, 25 units of 3-phosphoglycerate kinase, 0.45μM NADH, cell free extracts (3.1 or 3.46 mg protein), and water to 3.0 m*l* (Hohorst *et al.*, 1959).

The Entner-Doudoroff enzymes, 6-phosphogluconate dehydrase(6-phosphogluconate dehydratase, EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14), were assayed together by determining the amount of pyruvate formed from 6-phosphogluconate by the method of Keele, Hamilton, and Elkan (1969). The reaction mixture contained 0.1M tris-hydrochloride buffer(*p*H 7.6), 8mM gluconate-6-phosphate, 6mM $FeSO_4 \cdot 7\ H_2O$(freshly prepared), 3mM glutathione (freshly prepared), and extract and water to 1.0m*l*. $FeSO_4$ was required for maximum activity. The reaction mixture was incubated at 30°C for 60min and was stopped by the addition of 1 m*l* of 0.1 % solution of 2.4-dinitrophenylhydrazine in 2N hydrochloric acid. Pyruvate was determined by the modification of the method of Friedmann and Haugen (1943). A 1 m*l* sample was extracted with 3.0 m*l* of anhydrous ether. The ether phase was extracted with 6.0m*l* of 10% Na_2CO_3. After it was mixed, 5.0 m*l* of the lower phase was removed and mixed with 5.0 m*l* of 1.5 N NaOH. The enzymatic formation of pyruvate was confirmed by comparing the absorption spectra of the dinitrophenylhydrazone of a pyruvate standard to the test compound, as well as by comparing the optical density(OD) ratio (in nano-

mole) of both products (OD 490 — OD 540) (Smith and Gunsalus, 1957).

Aconitase[citrate(isocitrate)hydrolyase, EC 4.2.1.2] was assayed by the method of Anfinsen(1955) and was based on the spectrometric determination at 240nm of cis-aconitic acid. The reaction mixture contained 9.7mM DL-isocitrate, 48.3mM phosphate buffer (*p*H 7.4), and extract and water to 3.0 m*l*.

Fumarase (fumarate hydratase, EC 4.2.1.2) activity determinations were made with cell-free extracts by the method of Racker (1950), in which the production of L-malate from fumarate was followed by measuring the change in optical density to ultraviolet light (240nm) as fumarate is removed. The reaction mixture contained 0.05M phosphate buffer(*p*H 7.4), and cell-free extracts and water to 3.0 m*l*.

Isocitrate dehydrogenase [threo-DL-isocitrate:NADP(or NAD) oxidoreductase (decarboxylating), EC 1.1.1.41] was assayed by measuring the rate of NAD or NADP reduction at 340nm. The reaction mixture consisted of 10mM tris-hydrochloride (*p*H7.9), 0.33mM $MnCl_2$, 0.17mM DL-isotrate, 0.33mM NADP or NAD, and extract and water to 3.0m*l*.

Succinate dehydrogenase [succinate: (acceptor) oxidoreductase, EC 1.3.99.1] was assayed spectrophotometrically at 600nm after the reduction of 2,6-dichlorophenolindophenol(DCPIP), mediated by phenazine methosulfate(PMS), by the method of Arrigoni and Singer(1962). The reaction mixture consisted of 20mM succinate, 1mM KCN, 56mM phosphate buffer(*p*H 7.6), and cell-free extract and water to 3.0m*l*. The reaction mixture was incubated for 3min, and the reaction was initiated with 0.05mM DCPIP and 1.1mM PMS (Tabita and Lundgren, 1971).

The assay of α-ketoglutarate dehydrogenase[2-oxoglutarate: lipoate oxidoreductase(acceptor-acylating), EC 1.2.4.2] activity was based on the assay of Sanadi and Littlefield(1951) in which dichlorophenolindophenol reduction is followed spectrophotometrically at 600 nm. The assay mixture consisted of 33 mM phosphate buffer(*p*H 7.0), 6.67mM $MgCl_2$, 0.067mM thiamine pyrophosphate (TPP), 6.67mM α-ketoglutarate (*p*H 7.0), 0.033mM DCPIP, and extract and water to 3.0m*l*(Tabita and Lundgren, 1971).

NADH oxidase [NADH: (acceptor) oxidoreductase, EC 1.6.99.1] activity was measured by spectrophotometrically observing(340nm) the rate of NADH oxidation as described by Smith, London, and Stanier(1967) with some modification. The reaction mixture consisted of 0.25mM NADH, 66.7mM phosphate buffer (*p*H 7.4), and cell-free extract and water to 3.0m*l*.

The activity of each enzyme was determined under optimal conditions of assay (*p*H, concentration of substrates, cofactor, or activator) and was proportional to the amount of extract solution added to the reaction mixture. In all cases, except where noted, enzyme activity was expressed as mμ moles of substrates oxidized or reduced per milligram of protein per min.

Protein in the cell-free extracts was determined using the method of Lowry *et al.*(1951) with bovine serum albumin as a standard.

All chemicals and commercial enzyme

preparations were purchased from Sigma Chemical Co., St. Louis, Mo. U.S.A. All reagents were prepared in glass-distilled water.

RESULTS

The oxidation of thiosulfate in *T. concretivorus* was inhibited by various organic compounds. However, *T. concretivorus* oxidized the organic compound added as a sole source of energy (Hah and Ju, 1972). This result concludes that *T. concretivorus* utilizes some organic matter such as glucose and fructose. To confirm and extend metabolic pathways of organic substances, cell-free extracts of *T. concretivorus* were prepared and assayed for the key enzymes of the ED, the pentose shunt, EMP and TCA cycle.

Specific activities of glucokinase were 2 fold in heterotrophic cells (from thiosulfate-glucose medium) than that of autotrophic cells(from thiosulfate broth) (Fig. 1 and Table 1). That this difference is due to the repressive effect

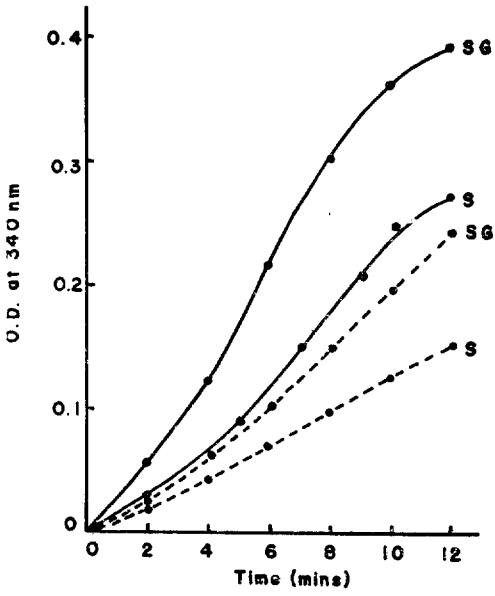

Fig. 1. Assay of hexokinase. Reaction mixture contained 15mM tris-buffer (*p*H 7.9), 2μM $MgCl_2$, 2.5μM glutathione, 0.25μM NAD, 1.25μM ATP, 5μM glucose or fructose, and crude enzyme and water to 3.0m*l*.

SG: cell-free extract from thiosulfate-glucose grown cells (3.1mg/m*l* protein).

S: cell-free extract from thiosulfate grown cells (3.46mg/m*l* protein).

(•———•) : glucose. (•·····•) : fructose.

Table 1. Enzymes of glucose metabolism in *Thiobacillus concretivorus*.

Enzyme	Specific activity(mμ mole/mg protein/min) of cell	
	Thiosulfate(0.5%)	Thiosulfate(0.5%)-glucose(0.5%)
Glucokinase	3.25	6.03
Phosphofructokinase	1.74	2.72
NADP-linked glucose-6-phosphate dehydrogenase	1.39	2.33
NAD-linked glucose-6-phosphate dehydrogenase	6.67	13.49
NADP-linked 6-phosphogluconate dehydrogenase	1.56	2.09
NAD-linked 3-phosphogluconate dehydrogenase	3.49	6.23
Fructose-1,6-diphosphate aldolase	3.31	4.67
NAD-linked 3-phosphoglyceraldehyde dehydrogenase	8.01	22.96
NADP-linked 3-phosphoglyceraldehyde dehydrogenase	0	0
6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase	0	0

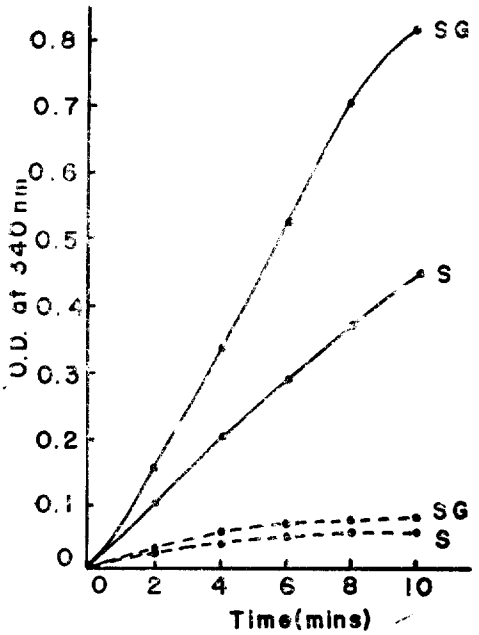

Fig. 2. Rate of NAD and NADP reduction by G-6-P dehydrogenase. Reaction mixture contained 15mM tris-buffer (*p*H 7.9), 6.67mM $MgCl_2$, 0.83mM G-6-P(disodium salt), 0.33 mM NAD (·——·)or NADP(······) (*p*H 7.0) and cell-free extract.
SG: Cell-free extract from thiosulfate-glucose grown cells (3.1 mg/m*l* protein).
S: Cell-free extrct from thiosulfate grown cells (3.46 mg/m*l*).

of thiosulfate is indicated by a comparison of their activities. These data also show the stimulatory effect of glucose on the synthesis of these enzymes.

Activities of G-6-P dehydrogenase, which catalyzed the initial reaction for both the pentosephosphate shunt and the ED pathway, were also increased 2 fold in the cells grown on glucose-containing media. Either NADP or NAD served as coenzyme for the G-6-P dehydrogenase from *T. concretivorus*, but specific activity of NAD-linked G-6-P dehydrogenase were much higher (6 fold) than that of NADP-linked G-6-P dehydrogenase in cells from all media. The NAD- and NADP-linked F-6-P dehydrogenase activities are probably due to two different enzymes since they vary independently (Fig. 2, Table 1).

Activities of 6-PG dehydrogenase were also increased by glucose. And the NAD-linked activity was 3 fold higher than the NADP-linked phosphogluconate dehydrogenase activity(Fig. 3 and Table 1). Phosphogluconate dehydrogenase and KDPG aldolase (ED enzymes) were assayed together by determining the amount of pyruvate formed from 6-phosphogluconate. The activities of ED enzymes were undetectable in all extracts tested. In *T. concretivorus* the failure to detect ED enzyme activities could be due either to the absence of the dehydrase, or the aldolase or both, since the two

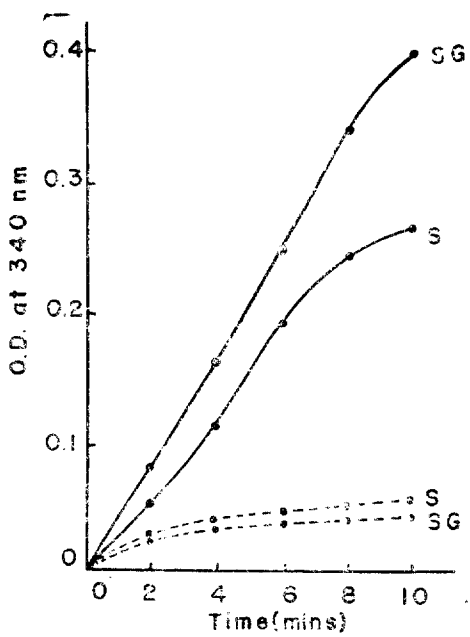

Fig. 3. Demonstration of 6-phosphogluconate dehydrogenase. The reaction mixture was the same as for glucose-6-phosphate dehydrogenase except substitution of 2.67mM 6-phosphogluconate for G-6-P.
NAD: (·——·), NADP:(······)

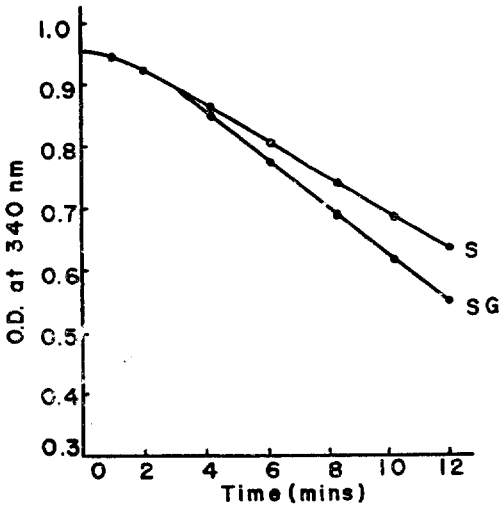

Fig. 4. Assay of fructose-1,6-diphosphate aldolase. The reaction mixture contained 0.1 M tris-buffer(*p*H 7.5), 5mM fructose-1,6-diphosphate, 0.25mM NADH (*p*H 7.0), 0.02 m*l* of α-glycerophosphate dehydrogenase-triosephosphate isomerase(10mg/m*l*) and cell extract and water to 3.0m*l*.
S:Thiosulfate grown cell-free extract (3.46 mg/m*l*).
SG:Thiosulfate-glucose grown cell-free extract (3.1mg/m*l*).

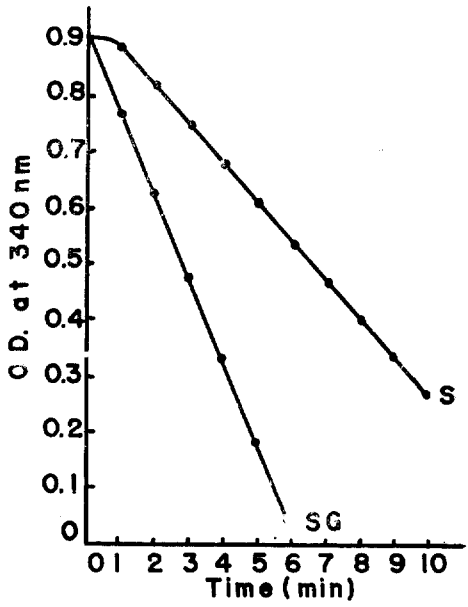

Fig. 5. Rate of NADH oxidation by 3-PGAL dehydrogenase. The reaction mixture contained 0.1M tris-buffer(*p*H 7.5), 10μM $MgCl_2$, 8μM glutathione, 10μM ATP, 10μM 3-phosphoglycerate, 25 units 3-phosphoglycerate kinase, 0.45μM NADH, and cell-free extract and water to 3.0m*l*.
S:Thiosulfate grown cell-free extract(3.46 mg/m*l* protein)
SG : Thiosulfate-glucose grown cell-free extract (3.1mg/m*l* protein)

enzymes were assayed jointly. Thus, this organism lacks the Entner-Doudoroff pathway(Table 1).

The specific activities of fructose-1, 6-diphosphate aldolase, an Embden-Meyerhof pathway enzyme, were low and showed relatively little variation in cells grown with and without glucose (Fig. 4, Table 1).

The dehydrogenase for glyceraldehyde -3-phosphate was NAD specific. The activity of the enzyme showed relatively high and was increased in the cells grown on thiosulfate-glucose media. It may be noted that the pattern of variation of this enzyme is similar to that of glucose-6-phosphate dehydrogenase(Fig. 5, Table 1)

In addition to the preceding enzyme, glucosephosphate isomerase was detected in extracts of *T. concretivorus*. The enzyme was measured with fructose-6-phosphate as substrate by coupling its activity with endogenous G-6-P dehydrogenase. The activities of glucosephosphate isomerase showed relatively high level in thiosulfate-glucose grown cells(Fig. 6, Table 1).

All activities of the key enzymes of glucose metabolism, except ED enzymes, were increased by growth in glucose-containing media. These differences in specific activities were interpreted as differences in amount of enzymes synthesized. The synthesis of those enzymes

was stimulated by growth in the presence of glucose as is shown by a comparison of their specific activities in cells grown in thiosulfate-glucose with those in cells from thiosulfate. These results indicate that the differences are due to the repressive effect of thiosulfate or the stimulatory effect of glucose on the synthesis of these enzymes.

In the glucose metabolism pathway of *T. concretivorus*, the activity of the pentose shunt enzyme, phosphogluconate dehydrogenase was relatively higher than that of EMP enzymes, and ED enzymes were lacking.

Five representative enzymes of the tricarboxylic acid cycle, plus NADH oxidase, were assayed in cell extracts prepared from thiosulfate-glucose grown cells.

The activities of the enzymes are shown in Table 2, and Fig. 7, 8, 9, and 10. Isocitrate dehydrogenase was present at high level in this organism. Either NAD or NADP served as coenzyme for the isocitrate dehydrogenase in *T. concretivorus*. NAD-linked isocitrate dehydrogenase is more active than the NADP-linked enzyme. Matin and Rittenberg(1971) and Hampton and Hanson

Table. 2. Activity of TCA cycle enzymes and reduced nicotinamide adenine dinucleotide oxidase.

Enzyme	Specific activity (μmole/mg protein/min)
NADP-linked isocitrate dehydrogenase	6.56
NAD-linked isocitrate dehydrogenase	17.49
Succinic dehydrogenase	13.12
α-ketoglutarate dehydrogenase	6.56
Aconitase	+
Fumarase	34.99
NADH oxidase	55.11

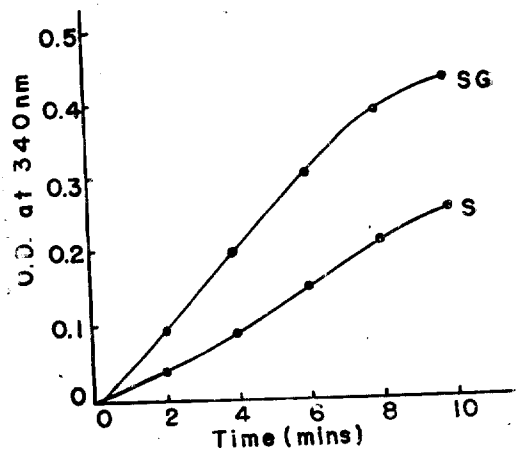

Fig. 6. Demonstration of phosphoglucoisomerase. The reaction mixture contained 15mM tris-buffer(pH 7.9), 2μM $MgCl_2$, 2.5μM glutathione, 0.25μM NAD, 5μM fructose-6-phosphate, and cell-extracts and water to 3.0ml.
SG : cell extract from thiosulfate-glucose grown cells. (3.1mg/ml protein)
S : cell extract from thiosulfate grown cells. (3.46mg/ml protein)

(1969) had reported that in the obligate chemolithotrophs NADP-linked enzyme could not be detected. In this experiment, the low activity of NADP-linked

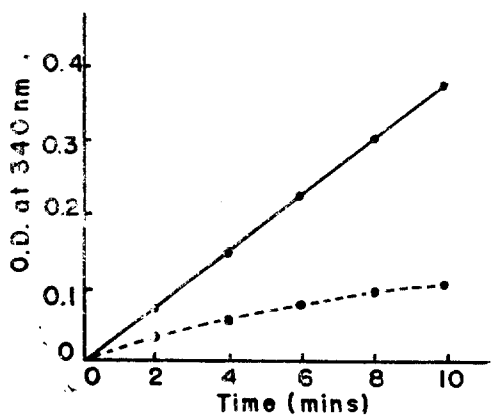

Fig. 7. Rate of NAD, NADP reduction by isocitric dehydrogenase. The reaction mixture consisted of 10mM tris-buffer(pH 7.9), 0.33mM $MnCl_2$, 0.17mM DL-isocitrate. 0.033 mM NADP or NAD, and extract and water to 3.0ml.
NAD : (·——·), NADP : (······)

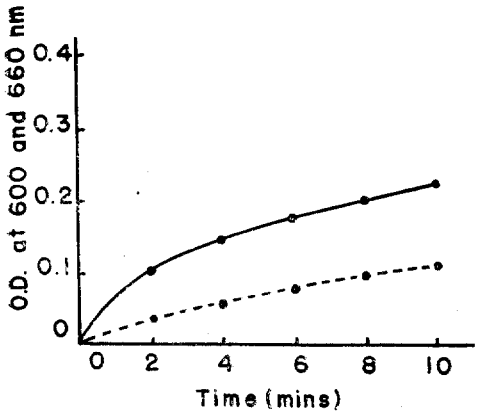

Fig. 8. Demonstration of α-ketoglutarate dehydrogenase and succinate dehydrogenase. The reaction mixture for the assay of α-ketoglutarate dehydrogenase contained 33mM phosphate buffer(*p*H 7.0), 6.67mM $MgCl_2$, 0.67mM TPP, 6.67mM α-ketoglutarate(*p*H 7.0), 0.033mM DCPIP, and extract(0.92mg protein) and water to 3.0m*l*. The reaction mixture for succinic dehydrogenase consisted of 20mM succinate, 1mM KCN, 56mM phosphate buffer(*p*H 7.69), and extract(0.92mg protein) and water to 3.0 m*l*.
Succinic dehydrogenase (•——•)
α-ketoglutarate dehydrogenase(•·····•).

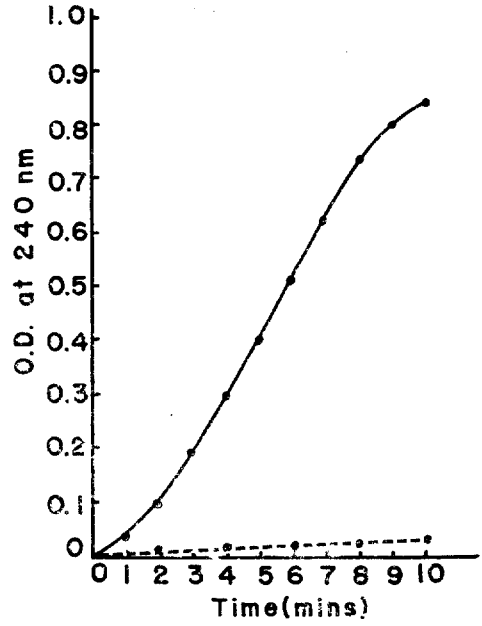

Fig. 9. Assay of aconitase and fumarase. The reaction mixture for aconitase consisted of 9.7mM DL-isocitrate, 48.3mM phosphate buffer(*p*H 7.4) and extract(0.92mg protein) and water to 3.0 m*l*. The reaction mixture for fumarase contained 0.05M sodium L-malate(*p*H 7.4), 0.05M phosphate buffer(*p*H 7.4), and cell-free extract(0.92mg protein) and water to 3.0 m*l*. aconitase (•·····•), fumarase (•——•)

isocitrate dehydrogenase was detected.

Specific activity of succinate dehydrogenase was also high. α-Ketoglutarate dehydrogenase also was present in the cells, but its activity was low. Although aconitase in cell extract was detected, its activity was very low. By this result, its presence is doubtful.

The specific activity of fumarase was highest in cell extracts. These results indicate that TCA cycle enzymes were operative.

The activity of NADH oxidase, which is implicated in the energy generating metabolism, was very high in the crude cell-free extract of *T. concretivorus*, recording 55.11mμ mole/min/mg protein. This well coincides with the fact that

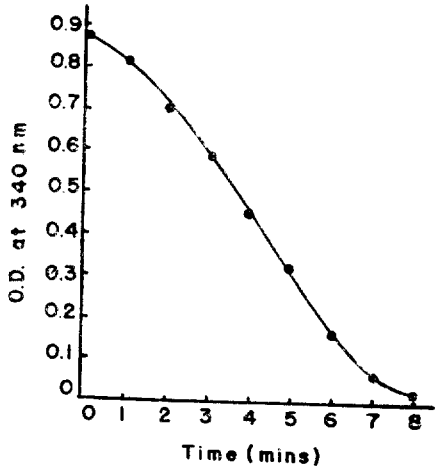

Fig. 10. Rate of NADH oxidation by NAD oxidase. The reaction mixture consisted of 0.25 mM NADH, 66.7mM phosphate buffer (*p*H 7.4), and cell free extract (0.92mg protein) and water to 3.0 m*l*.

activities of NAD-linked G-6-P dehydrogenase, 6-PG dehydrogenase and 3-PGAL dehydrogenase were high.

DISCUSSION

Thiobacillus concretivorus is the chemolithotroph which is capable of oxidizing dissimilar inorganic substrates for energy-reduced sulfur compounds. This organism is able to utilize organic compounds such as glucose with thiosulfate (Hah *et al.*, 1972; and Hah and Ju, 1972). The change in growth substances causes a profound effect on both sulfur oxidation (Hah and Ju, 1972) as well as CO_2 fixtion; the latter was tested by determining repression of the enzyme ribulose-1,5-diphosphate carboxylase (Hah *et al.*, unpublished data). Otherwise the growth substrate also affects the specific activities of catabolic enzymes in cells.

The present results based upon enzyme studies support the conclusion that the pentose phosphate shunt pathway is the major route of glucose dissimilation by *T. concretivorus*. This conclusion based upon following evidences.

The first is the relatively high specific activities of NAD-linked G-6-P dehydrogenase, 6-phosphogluconate dehydrogenase and glucokinase, as compared to the specific activities of enzymes unique to the EMP in cells grown in thiosulfate or thiosulfate-glucose broth. Activities of pentose shunt pathway enzymes are enhanced by glucose in thiosulfate-glucose media, where glucose is metabolized and provides the organism with energy. No similar relation was observed by the EMP enzymes.

The second is that the failure to detect ED enzymes in cells grown in thiosulfate or thiosulfate-glucose medium eliminates the ED pathway as a significant route of glucose catabolism in *T. concretivorus*. Matin and Rittenberg (1971) reported that *T. novellus*, *T. neapolitanus*, *T. thiooxidans* and *T. thioparus* lacked ED pathway because of the absence of 6-phosphogluconate dehydrase. The absence of the dehydrase has also been reported in glucose-grown *E. coli*, *Enterobacter aerogenes*, and *Salmonella typhimurium* (Eisenberg and Dobrogosz, 1967) and *Micrococcus sodonensis* (Perry and Evans, 1967), in glucose- or gluconate-grown *Erwinia carotovora*, *Serratia marcescens* (Eisenberg and Dobrogosz, 1967), and in *Brucella abortus* (Robertson and McCullough, 1968), the wild type strain of *Rhodopseudomonas spheroides* (Szymona and Doudoroff, 1960).

Third, it is the pentose phosphate pathway and related enzymes whose syntheses are responsible for the presence of glucose in the growth environment. In contrast to the wide variations in specific activities of these enzymes, the enzymes unique to the EMP such as F-1,6-Dip. aldolase are present at low and relatively constant levels in cells grown under all conditions tested. For the reason, EMP is likewise eliminated as a major route of glucose catabolism in *T. concretivorus*. It appears, therefore, that the role of EMP is probably functioning as a subway of carbohydrate metabolism.

When the pentose phophate pathway and Embden-Meyerhof pathway are present simultaneously, glucose-6-phosphate is a critical branch point. By con-

trolling the activity of glucose-6-phosphate dehydrogenase, the organism can regulate the flow of glucose into one or the other of these pathways. According to Matin and Rittenberg (1971), its inhibition by ATP suggests that the imposition or relief of inhibition of this enzyme is a key regulatory process.

The enzymes essential for the utilization of glucose *via* the pentose phosphate pathway are induced or repressed by the presence in the nutritional milieu of glucose or thiosulfate respectively. The concentration of substrate influences the level of enzymes such as G-6-P dehydrogenase.

Since the specific activity of phosphoglyceraldehyde dehydrogenase (NAD-specific) in thiosulfate-glucose grown cells is high, it is possible that phosphoglyceraldehyde generated from glucose is converted mainly into pyruvate which is channeled into the TCA cycle. Smith *et al.*(1967) showed that, in contrast to the facultative autotrophs *T. intermedius* and *Hydrogenomonas eutropha*, the obligate autotrophs *Anacystis midulans*, *Coccochloris peniocystis*, *T. thiooxidans*, and *T. thioparus* lacked the enzymes α-ketoglutarate dehydrogenase and NADH oxidase. The absence of the α-ketoglutarate dehydrogenase has also been reported in *T. thermophilica* (Williams and Hoare, 1972) and *Nitrosomonas* (Hooper, 1969). These suggested that the TCA cycle merely serves a biosynthetic function in chemoautotrophs.

This is somewhat unexpetced since most biosynthetic enzymes generally are linked to NADP rather than to NAD (Klingenberg and Slenczka, 1959). Several authors, however, have found that α-ketcglutarate dehydrogenase and NADH oxidase are present in extracts from several chemolithotrophs (Butler and Umbreit, 1969; Matin and Rittenberg, 1970; Smith and Hoare, 1968; Trudinger and Kelly, 1968; Hooper, 1969; Tabita and Lundgren, 1971; Hempfling and Vishniac, 1965).

All enzymes (aconitase is doubtful) of the TCA cycle tested and NADH oxidase were detected in the cells of *T. concretivorus*. The presence of two isocitrate dehydrogenases (NAD- and NADP-linked) may have important regulatory functions for this organism.

These results support the conclusion that TCA cycle in this organism is functioning in both biosynthetic and energy regulating reactions.

The activity of NADH oxidase was very high in the crude cell-free extract of *T. concretivorus*, recording 55.11mμ mole/min/mg protein. This well coincides with the fact that activities of NAD-linked G-6-P dehydrogenase, 6-PG dehydrogenase and 3-PGAL dehydrogenase were high. Tabita and Lundgren (1971) suggested that the activity of NADH oxidase was high in *T. ferrooxidans* grown in thiosulfate-glucose since this enzyme was inducible by glucose.

Although *T. concretivorus* and other obligate autotrophs involved enzymes of pentose phosphate, Embden-Meyerhof pathway and TCA cycle, they do not utilize organic substance as sole source of energy. The mechanism is not dissolved up to date. Although Smith *et al.* (1967) proposed the absence of α-ketoglutarate dehydrogenase or NADH

oxidase as the possible mechanism, it became apparent by this study that the reason is not the absence of these enzymes, but some others still uncertain.

The presence of an inducible pentose phosphate pathway in *T. concretivorus*, *T. thiooxidans*, *T. thioparus*, and *T. neapolitanus* (Matin and Rittenberg, 1971) when considered with other common physiological features such as predominance of NAD-linked dehydrogenases (Matin and Rittenberg, 1970; Hah *et al.*, 1972) and morphological features, suggest a close taxonomic relationship between these organisms.

Hutchinson *et al.* (1966) asserted that *T. concretivorus* and *T. thiooxidans* are the same species. However, on the results of these studies, the two species are apparently different in their enzymes involved (NAD- and NADP- linked enzymes being in *T. concretivorus*, but only NAD-linked in *T. thiooxidans*) and enzyme properties (enzymes in *T. concretivorus* being inducible by glucose but in *T. thiooxidans* not).

摘 要

Thiosulfate 및 thiosulfate와 glucose를 添加한 培地에 各各 기른 *T. concretivorus*로 부터 粗酵素를 抽出하여 炭水化物代謝에 관여하는 數種 要石酵素의 活性을 測定하여 다음과 같은 結果를 얻었다.

1. Pentose Shunt經路에 관여하는 酵素 즉 glucokinase, G-6-P dehydrogenase, 6-PG dehydrogenase 및 phosphoglucoisomerase의 活性은 各各 6.03, 13.49, 6.23 mμ mole/min/mg protein으로서 EMP에 관여하는 酵素의 活性보다 높았다. Pentose Shunt에 관여하는 酵素의 生産 및 活性이 培地의 glucose에 의하여 促進되나 EMP의 그것은 별로 큰 영향을 받지 않았다. 그리고 ED經路에 관여하는 酵素인 KDPG aldolase가 검출되지 않았다. 따라서 *T. concretivorus*에 있어서는 Pentose Shunt經路가 energy生産의 主된 役割을 하고 EMP經路는 subway的 役割을 하며 ED經路는 없는것으로 판단되었다.

2. G-6-P와 6-PG dehydrogenase는 cofactor로 NAD와 NADP를 要求하는 두가지 種類가 있으며, 이중 NAD를 要求하는 酵素의 活性이 NADP를 要求하는 酵素보다 2倍나 높다. 그러나 3-PGAL dehydrogenase는 NAD를 要求하는 효소만이 存在하였으며 本菌에 있어서의 活性이 22.96으로서 대단히 높았다.

3. TCA cycle을 밝히는데 要石이 되는 모든 酵素와 NADH oxidase가 本菌에서 검출되었다. 이들중 fumarase와 isocitrate 및 succinate dehydrogenase의 活性은 各各 34.99와 17.49 및 13.12로서 대단히 높았으며 여타 酵素의 活性은 낮았다. Isocitrate dehydrogenase는 cofactor로서 NAD와 NADP를 要求하는 두 種類가 存在하였다.

4. 本菌의 粗酵素 중에는 energy發生에 관여하는 NAD-oxidase의 活性이 55.11 mμ mole/min/mg protein으로서 대단히 높았다. 이는 NAD-linked G-6-P와 6-PG dehydrogenase 및 3-PGAL dehydrogenase의 活性이 높은 것과 아주 잘 一致한다.

5. Obligate chemolithotrophic bacteria에 있어서는 存在하지 않는다는 α-ketoglutarate dehydrogenase와 NADH oxidase 및 NADP-linked isocitrate dehydrogenase가 本菌에서는 存在하였다.

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