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Constitutive Nature of 5-Ketogluconate Reductase of Gluconobacter Suboxydans

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CONSTITUTIVE NATURE OF 5-KETOGLUCONATE REDUCTASE OF *GLUCONOBACTER SUBOXYDANS*

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CONSTITUTIVE NATURE OF 5-KETOGLUCONATE REDUCTASE

OF GLUCONOBACTER SUBOXYDANS

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OF *GLUCONOBACTER SUBOXYDANS*

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$NADP^+$-dependent 5-ketogluconate reductase (D-Gluconate:$NADP^+$ oxidoreductase; E.C.1.1.1.69.) of *Gluconobacter suboxydans* was examined and determined to be constitutive in nature. Crude, cell-free enzyme extracts were prepared by sonication of cells grown on 2.0% glucose and 2.0% ethanol in various experiments. The specific activity of 5-keto-gluconate reductase in the crude enzyme extracts of cells grown under various conditions was determined. The specific activity remained relatively constant strongly indicating the constitutive nature of the enzyme.

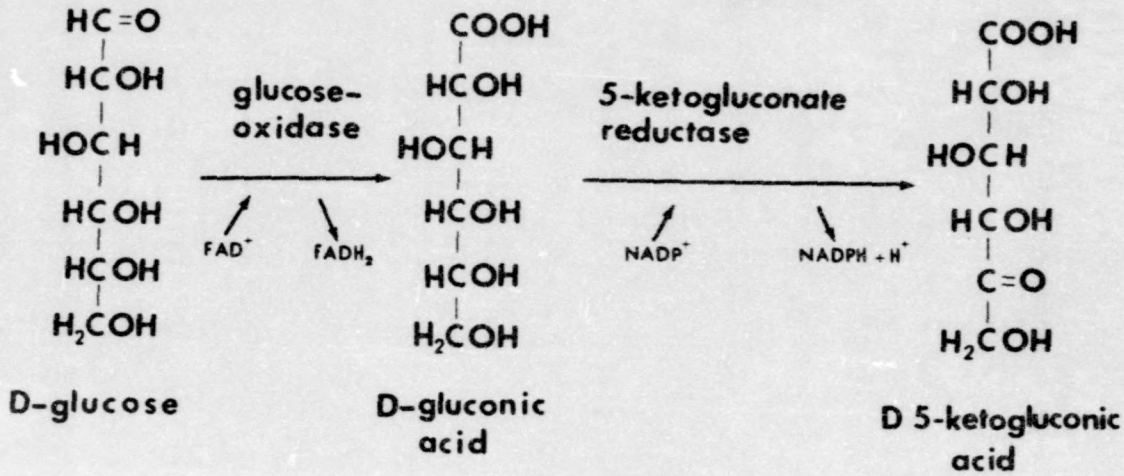
INTRODUCTION

Gluconobacter suboxydans (Kluyver and DeLeew) De Ley and Frateur is a chemoorganotroph which carries out aerobic respiration (4). G. suboxydans metabolizes ethanol to acetic acid, and polyhydroxy alcohols are converted to ketones and in some instances may be further metabolized to acids. When D-glucose is the sole source of carbon and energy, G. suboxydans will convert this sugar to gluconic acid (GA) and subsequently 5-ketogluconic acid (5-KGA) (Fig. 1). The oxidation of glucose is catalyzed by the enzyme glucose oxidase, and gluconic acid oxidation is catalyzed by $NADP^+$-linked 5-ketogluconate reductase (5-KGR) (5, 8, 9). Further metabolism of 5-KGA will result in the formation of tartaric acid (12).

5-ketogluconic acid reductase has been detected in a variety of microorganisms, such as Klebsiella (6), Escherichia (6), and Pseudomonas (4), in addition to G. suboxydans (8, 10). The enzyme catalyzes the dehydrogenation of the hydroxyl group of the number five carbon of D-gluconic acid in accordance with Bertrand's rule for the dehydrogenation of polyhydroxy alcohols (10, 12).

De Ley and Stouthamer (7) have reported that gluconic acid is converted to 5-KGA by a $NADP^+$-linked reductase in a ketogenic process in G. suboxydans. Ameyama, Chiyonobu, and Adachi (2, 3) using Gluconobacter liquefaciens have presented data which indicates that 5-KGA is the only keto acid produced, suggesting that 5-KGR is a major ketogenic enzyme in this process. Also, Okamoto (14) studying glucose metabolism of G. suboxydans has reported a 90% yield of 5-KGA.

Figure 1. Steps in conversion of D-glucose to D 5-ketogluconic acid.


HC=O
HCOH
HOCH
HCOH
HCOH
H2COH
D-glucose
glucose-oxidase
FAD+
FADH2
COOH
HCOH
HOCH
HCOH
HCOH
H2COH
D-gluconic acid
5-ketogluconate reductase
NADP+
NADPH + H+
COOH
HCOH
HOCH
HCOH
C=O
H2COH
D 5-ketogluconic acid

Although the enzymatic conversion of gluconic acid has been a point of interest of many investigators, no extensive data presently exist concerning the purification and biochemical properties of this enzyme. Okamoto (13) made the first analytical attempt to purify the enzyme and obtained a 9-fold purification of a cell-free enzyme extract from G. suboxydans. Ameyama et al. (2) reported a 120-fold purification of 5-KGR from the soluble fraction of cells of G. liquefaciens.

A point of interest in enzymatic studies involves the factors affecting enzyme synthesis in microorganisms. The occurrence and concentration of enzymes in cells under different metabolic conditions are variable. Enzyme induction is a process by which the level of concentration of the enzyme is increased when the substrate is added to the culture medium. On the other hand a constitutive enzyme is present in nearly constant concentration regardless of the presence or absence of its specific substrate (11). Aiba (1) has stated that production of 5-KGA from GA by G. suboxydans utilizing glucose is enzymatically an inducible process, and 5-KGR is not produced until all of the glucose in the culture medium has been converted to gluconic acid. It has been reported that 5-KGR is also inducible in Klebsiella and Escherichia (8). Most species of Gluconobacter will convert GA to 5-KGA to some degree. The objective of this work was to ascertain whether 5-KGR of G. suboxydans is a constitutive enzyme or an inducible enzyme.

MATERIALS AND METHODS

Chemicals

$NADP^+$ (monopotassium salt), D-gluconic acid, and sodium carbonate were obtained from Sigma Chemical Co. Reagent grade monobasic and dibasic potassium phosphate, glycine (ammonia free), sodium citrate dihydrate, cupric sulfate pentahydrate, potassium iodate, potassium iodide, and oxalic acid were purchased from Matheson, Coleman, and Bell Chemical Co. Sodium hydroxide and sodium bicarbonate were purchased from Allied Chemical Co. Calcium carbonate was obtained from Baker Chemical Co. Yeast extract and Lintener's soluble starch were obtained from Difco Chemicals, Inc. Commercial grade glucose monohydrate was obtained from CPC International, Inc.

Source of the enzyme

Gluconobacter suboxydans ATCC 621 was used throughout this investigation. Stock cultures of G. suboxydans were maintained on mannitol agar slants with the following composition per liter: mannitol, 20 gm; yeast extract, 2.5 gm; agar, 2.1% (w/v). Mannitol slants were inoculated with the organism and incubated at 28 C for approximately two days. The stock cultures were stored at 6 C.

Culture media containing 20 gm of mannitol and 2.5 gm yeast extract per liter were prepared for the inoculum. The media were dispensed in 250-ml Ehrlenmeyer flasks in 150 ml quantities and inoculated from the stock slant cultures. Ordinarily two loopfuls (2.0 mm in diameter) were

used. The culture flasks were incubated for 18-22 hours at 28 C employing a New Brunswick Scientific Company Rotary Shaker set at 240 rpm.

Experimental growth media which contained either glucose (2.0%, w/v) or ethanol (2.0%, v/v), yeast extract (0.25%, w/v) and $CaCO_3$ (1.0%, w/v) were dispensed in 1.0-l Ehrlenmeyer flasks in 250-ml quantities. Each culture was inoculated with 10 ml of the inoculum. The flasks were placed in a New Brunswick Scientific Company Controlled Environment Incubator Shaker (180 strokes per min) and incubated at 28 C for an appropriate incubation period.

The cells were collected by centrifugation at 27,000 x g for 15 min using a Sorvall Superspeed Rc2-B Automatic Refrigerated Centrifuge with a Sorvall SS-34 rotor. After decanting the supernatant fluid, the pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.0). The cell suspension was centrifuged at 27,000 x g for 5 min, and the cells were resuspended in a buffer of the same composition after the supernatant fluid had been decanted. The cells were disrupted by sonication using a Branson Sonic Cell Disruptor set at 60 watts for 7 min. The cell debris was removed by centrifugation at 43,500 x g for 20 min at 3 C, and the resulting supernatant fluid was used as the enzyme source.

Assay of enzyme activity

The activity of 5-ketogluconate reductase (D-Gluconate:$NADP^+$ oxidoreductase; E.C.1.1.1.69) was determined by following the reduction of $NADP^+$ at 340 nm in a Bausch and Lomb Spectronic 505 Recording Spectrophotometer.

The reaction mixture for assay of 5-ketogluconate reductase activity contained 0.05 M glycine/NaOH buffer (pH 9.5), water, enzyme (crude extract),

0.01 M $NADP^+$ and 0.01 M gluconic acid in a final volume of 1.0 ml. Gluconic acid (sodium salt) was used to start the reaction. One unit of enzyme was defined as the amount of enzyme required to reduce 1.0 µmole of $NADP^+$ per minute. Specific activity was expressed as the ratio of units of enzyme per milligram of protein (8).

Protein assay

The concentration of protein in the crude extract was determined by Waddell's method as modified by Kies (16). A Bausch and Lomb Precision Spectrophotometer was employed to measure absorbancy.

Quantitative determination of reducing sugars

The concentration of glucose and 5-ketogluconic acid in the culture media was determined by the method of Shaffer and Hartman (15).

RESULTS AND DISCUSSION

pH Optimum

The pH optimum for this enzyme was determined using two buffer systems; namely, 0.1 M potassium phosphate buffer (pH 4.5 to 8.5) and 0.1 M glycine/NaOH buffer (pH 7.0 to 10.5) (Fig. 2). The optimal pH for 5-KGR activity of mannitol-grown cells was pH 9.5 in 0.1 M glycine/NaOH buffer.

Activity of 5-ketogluconate reductase from mannitol-grown cells of Gluconobacter suboxydans

Cells were grown in culture media containing 2.0% (w/v) mannitol as the sole carbon and energy source, and cell-free enzyme extracts were prepared. These extracts were found to contain 5-KGR activity. This observation indicated the presence of 5-ketogluconate reductase in the actively growing cells of G. suboxydans regardless of the presence or absence of the gluconic acid substrate.

Specific activity of 5-ketogluconate reductase from glucose-grown cells of Gluconobacter suboxydans

The data presented in Figure 3 was obtained from a time-lapse experiment in which G. suboxydans was grown on 2.0% (w/v) glucose as the sole source of carbon and energy. The inoculum was prepared as previously described. A number of 1.0-l Ehrlenmeyer flasks, each containing 250 ml of culture growth medium, were inoculated with cells of G. suboxydans and placed on the incubator shaker set at 28 C. After 12 hours of incubation,

Figure 2. The effect of pH on the activity of 5-ketogluconate reductase of Gluconobacter suboxydans utilizing gluconic acid as the substrate.

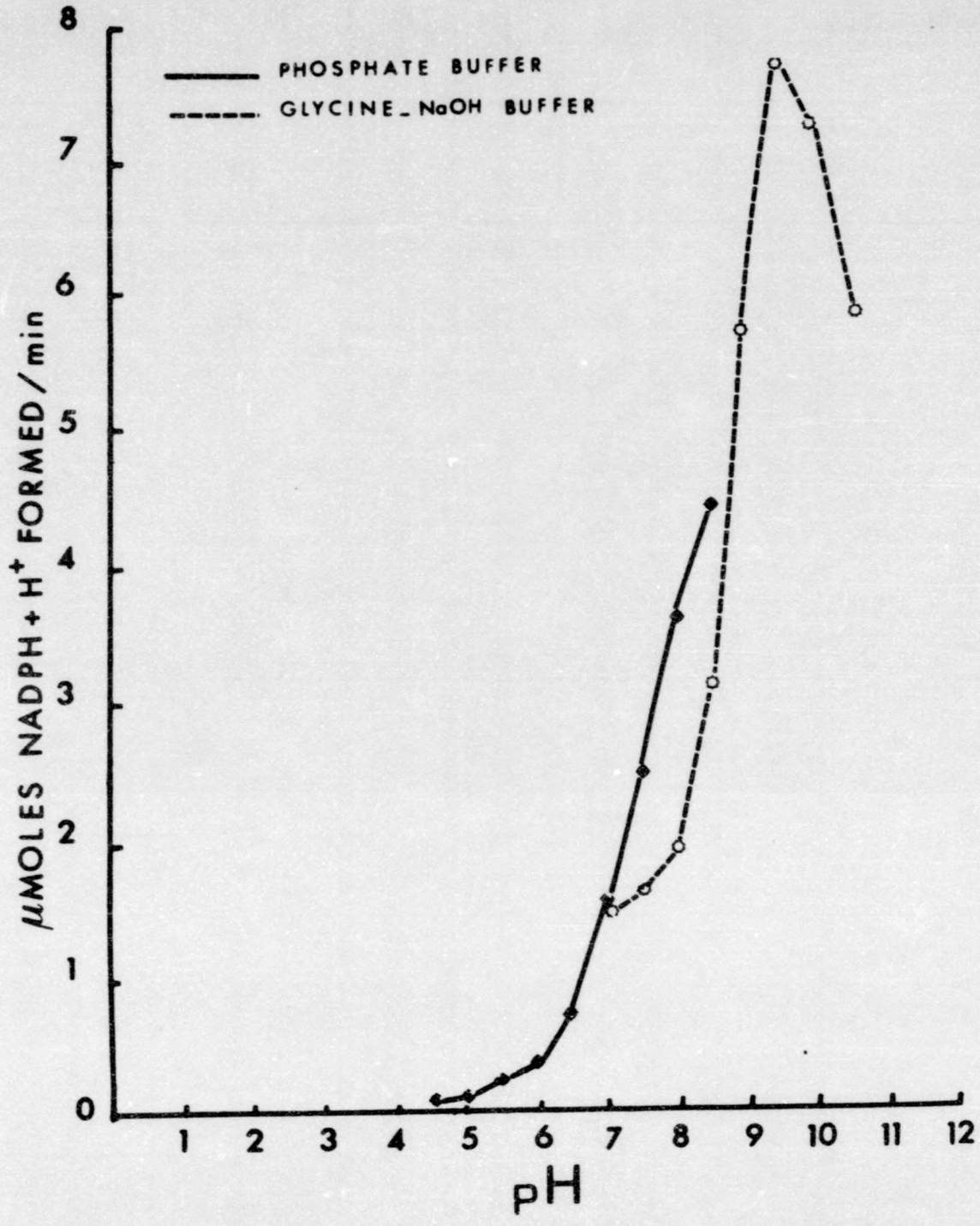
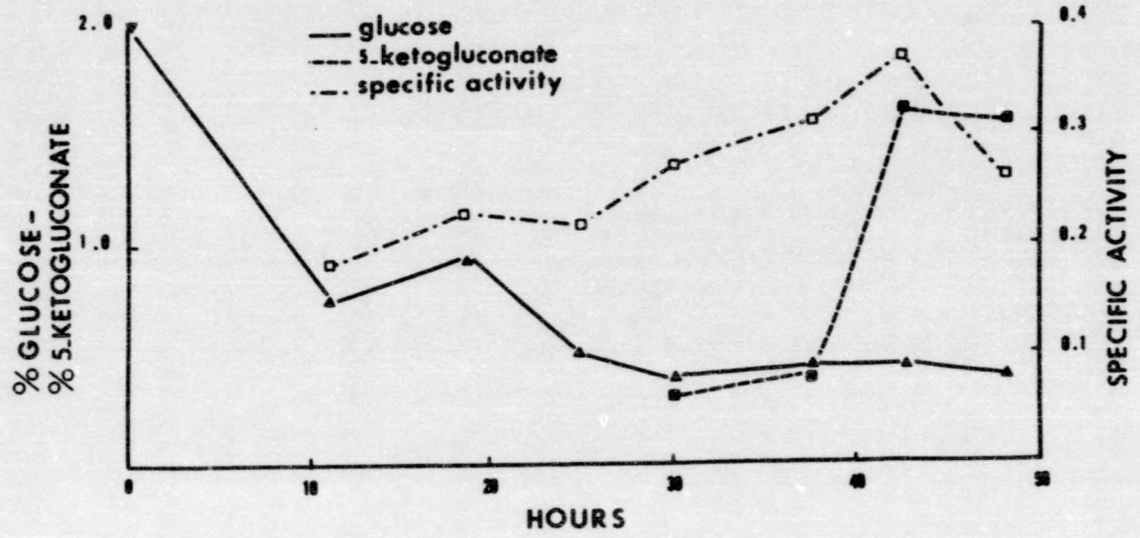

PHOSPHATE BUFFER
GLYCINE_NaOH BUFFER
μMOLES NADPH + H+ FORMED / min
0
1
2
3
4
5
6
7
8
1
2
3
4
5
6
7
8
9
10
11
12
pH

Figure 3. The effect of incubation time on the specific activity of 5-ketogluconate reductase of glucose-grown Gluconobacter suboxydans.


glucose
5-ketogluconate
specific activity
% GLUCOSE -
% 5-KETOGLUCONATE
SPECIFIC ACTIVITY
HOURS
2.0
1.0
0.4
0.3
0.2
0.1
0
10
20
30
40
50

two cultures were removed, and the specific activity of the enzyme was determined from the cells of one culture. The concentration of glucose and 5-ketogluconic acid in the second culture was determined concommitantly. The above procedure was repeated at 18, 24, 30, 36, 42, and 48 hours. Upon consumption of glucose by the cells of G. suboxydans in the growth media, GA was initially formed and subsequently 5-KGA was formed from GA. However, the specific activities remained relatively constant over a period of 20 hours. The small increase in specific activities between 24 and 42 hours was insignificant.

The effect of glucose additions on the specific activity of 5-ketogluconate reductase from glucose-grown cells of Gluconobacter suboxydans

The data in Figure 4 were obtained from an experiment in which 2.0% (w/v) glucose was the sole source of carbon and energy. Furthermore, additions of glucose (final concentration ca. 1.0%, w/v) were made following 36, 42, 54, and 65 hours of incubation. The low specific activity at 42 hours may have been the result of cellular death due to starvation as nutrients were depleted from the culture media. However, after the glucose additions were made, the specific activity reached a constant level and remained as such until glucose concentration was diminished at 90 hours.

Specific activity of 5-ketogluconate reductase from ethanol-grown cells of Gluconobacter suboxydans

The data presented in Figure 5 were obtained from an experiment in which 2.0% (v/v) ethyl alcohol was the sole source of carbon and energy. The specific activities of the enzyme extracts remained constant over a period of 60 hours at which time the experiment was terminated. Ethanol is oxidized to acetic acid by G. suboxydans (4); therefore, in the absence of

Figure 4. The effect of additions of glucose at intervals on the specific activity of 5-ketogluconate reductase of glucose-grown Gluconobacter suboxydans

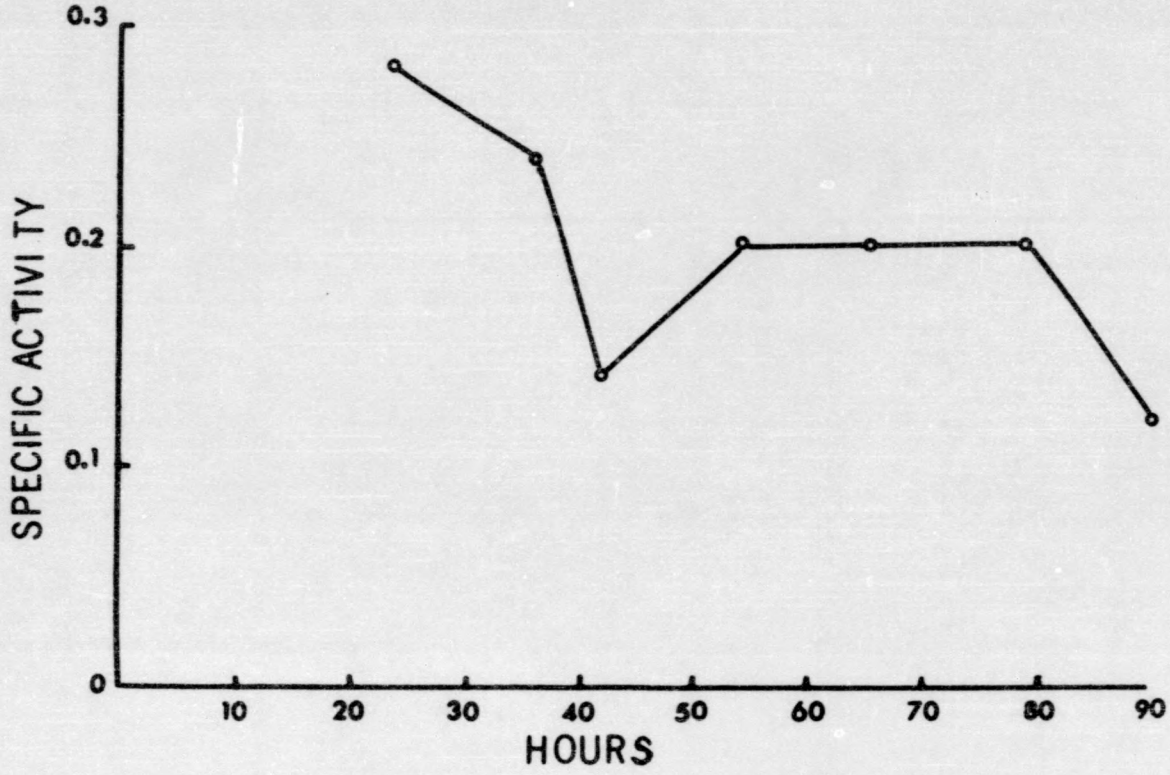
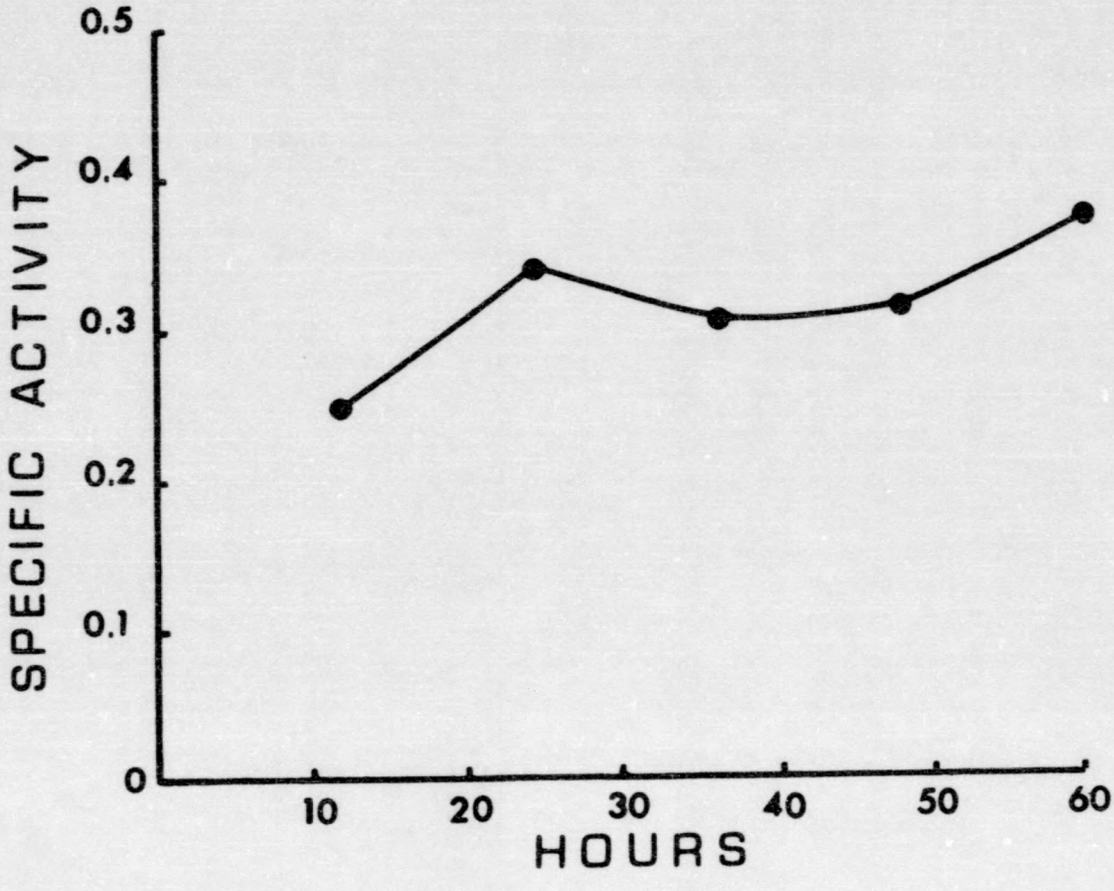

0.3
0.2
0.1
0
SPECIFIC ACTIVITY
10
20
30
40
50
60
70
80
90
HOURS

Figure 5. The specific activity of 5-ketogluconate reductase isolated from ethyl alcohol-grown cells of Gluconobacter suboxydans harvested at 12-hour intervals.


SPECIFIC ACTIVITY
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
HOURS

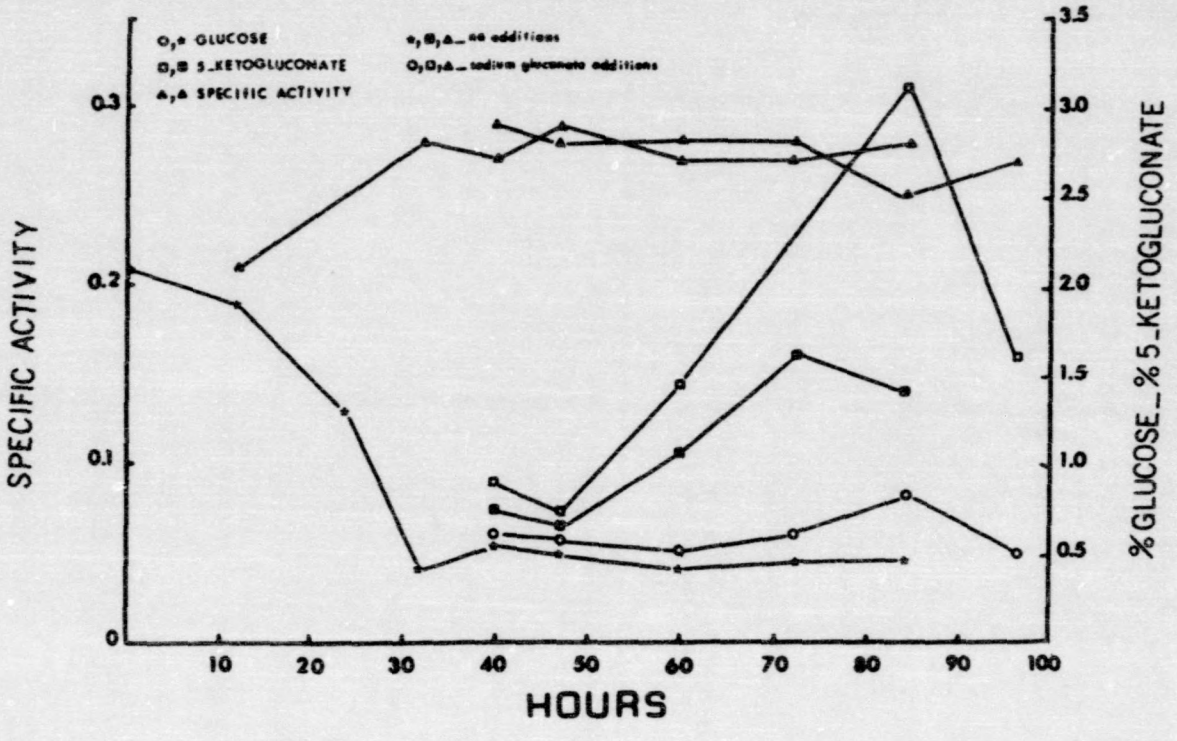
glucose, gluconic acid is not synthesized by the cells. However, the specific activities remained within the range reported previously (Figs. 3, 4). These data support the conclusion regarding the constitutive nature of 5-ketogluconate reductase of G. suboxydans.

The effect of gluconic acid additions on the specific activity of 5-ketogluconate reductase from glucose grown-cells of Gluconobacter suboxydans

The data presented in Figure 6 were obtained from an experiment in which gluconic acid was added to one series of cultures and omitted from another series. In all cultures, 2.0% (w/v) glucose was used as the carbon and energy source. Additions of gluconic acid (final concentration ca. 1.0%, w/v) were made at intervals of 32, 40, 47, 60, and 72 hours to one series of cultures and the other series retained as a control. In this experiment, the specific activities and concentrations of glucose and 5-ketogluconic acid were determined concommitantly. Higher concentrations of 5-KGA were detected in those cultures to which gluconic acid had been added (Fig. 6). The specific activities from both series of cultures remained constant over a period of 64 hours, beginning at hour 32 and ending at hour 96. In spite of higher accumulations of 5-KGA, no noticeable differences could be seen in the range of specific activities obtained. In comparison, the lower accumulations of 5-KGA observed in cultures to which no additions were made reveal no apparent variation in the range of specific activities.

The specific activities of 5-ketogluconate reductase obtained from the cell-free enzyme extracts of cultures of G. suboxydans grown under various conditions described in this investigation have remained within

Figure 6. A comparison of specific activity of 5-ketogluconate reductase from glucose-grown cells of Gluconobacter suboxydans with and without gluconic acid additions.


O,* GLUCOSE
□,■ 5-KETOGLUCONATE
Δ,▲ SPECIFIC ACTIVITY
*,■,▲ no additions
O,□,Δ sodium gluconate additions
SPECIFIC ACTIVITY
%GLUCOSE-%5-KETOGLUCONATE
HOURS
0
0.1
0.2
0.3
0.5
1.0
1.5
2.0
2.5
3.0
3.5
10
20
30
40
50
60
70
80
90
100

the narrow range of 0.2 to 0.3. The uniformity of these results indicates the constitutive nature of 5-ketogluconate reductase. The results of this investigation differ from those reported by Aiba (1) in which an inducible process for conversion of gluconic acid to 5-ketogluconic acid by the 5-ketogluconate reductase of G. suboxydans was suggested.

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