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Electrophoretic Heterogeneity of Nicotinamide Adenine Dinucleotide Phosphate-Dependent 5-Ketogluconate Reductase of Gluconobacter Suboxydans

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ELECTROPHORETIC HETEROGENEITY OF NICOTINAMIDE ADENINE

DINUCLEOTIDE PHOSPHATE-DEPENDENT 5-KETOGLUCONATE

REDUCTASE OF GLUCONOBACTER SUBOXYDANS

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TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

ELECTROPHORETIC HETEROGENEITY OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-DEPENDENT 5-KETOGLUCONATE REDUCTASE OF GLUCONOBACTER SUBOXYDANS

Sharon L. Dianis   July 1976   27 pages

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NADP$^+$-dependent 5-ketogluconate reductase (D-Gluconate:NAD(P)$^+$

oxidoreductase E.C. 1.1.1.69) isozymes were detected in cell-free extracts

of Gluconobacter suboxydans (American Type Cultures Collection, Bethesda,

Maryland, strain 621). Cell-free enzyme extracts were prepared from cells

grown in media which contained glucose, gluconate, glycerol, sorbitol,

or mannitol as the sole carbon source in 2% concentration. The extracts

were fractionated using analytical polyacrylamide disc-gel electrophoresis.

Gels were stained specifically for 5-ketogluconate reductase. Three

isozymes were present in each extract from cells grown on the five different

carbon sources. Gels stained in a reaction mixture which contained glycerol

exhibited two of the isozymes.

An attempt was made to separate the three isozymes using anion-exchange

chromatography. A stepwise anion-exchange procedure on DEAE Sephadex re-

sulted in the separation of one of the isozymes from the other two and indi-

cated that this isozyme might actually be two isozymes with the same electro-

phoretic mobility. Results from linear gradient anion-exchange chromatography

disproved this possibility.

A comparison of results obtained from fractionation by linear gradient

anion-exchange of extracts which had been previously fractionated on

Sephadex G-100 gel and those which had not, indicated that gel filtration

did not improve separation of the isozymes.

INTRODUCTION

The metabolism of glucose by the acetic acid bacteria proceeds through direct non-phosphorylative oxidation or by phosphorylative oxidation. Acetic acid bacteria are classified in two genera, Gluconobacter and Acetobacter. One of the bases for this classification is their method of carbohydrate metabolism. Acetobacter species produce acetic acid from ethyl alcohol whereas Gluconobacter species produce little, if any, acetic acid. Gluconobacter species metabolize most of the glucose present in the medium to gluconic acid while Acetobacter species do not. Kitos et al. (1958) found that in Acetobacter suboxydans, the majority of the glucose was metabolized by the pentose phosphate pathway after phosphorylation. When the cells were grown aerobically, 63% of the glucose in the medium entered the pentose phosphate pathway. All of the glucose which was not metabolized via the pentose phosphate pathway underwent direct non-phosphorylative oxidation.

Whiting, Midgley, and Dawes (1975) have found both of these pathways in Pseudomonas aerugenosa. They found that the enzymes of the direct oxidative pathway are located in the cytoplasmic membrane, and glucose is oxidized extracellularly. The products of the oxidation enter the cell via a specific transport system. Any glucose which enters the cell intact is phosphorylated and metabolized by either the Enter-Doudoroff pathway or the pentose phosphate pathway.

1

The direct non-phosphorylative oxidation of glucose by species of Gluconobacter has been partially elucidated by Kodama, Kotera, and Yamado (1972). Glucose is first oxidized to gluconic acid by the enzyme glucose oxidase. Gluconic acid is then dehydrogenated to form either 2-ketogluconic acid or 5-ketogluconic acid. Two specific dehydrogenases are involved in this conversion. An $NADP^+$-dependent 2-ketogluconate reductase catalyzes the conversion of gluconic acid to 2-ketogluconic acid, and $NADP^+$-dependent 5-ketogluconate reductase activity results in 5-ketogluconic acid formation. The 5-ketogluconic acid may then be converted to L-tartaric acid and glycolic acid. It was determined by Stubbs et al. (1940) that 2-ketogluconate reductase is active during the oxidation of glucose to gluconic acid by the glucose oxidase. The 5-ketogluconate reductase was reported not to actively catalyze the conversion of gluconic acid to 5-ketogluconic acid until all of the glucose had been oxidized.

It is economically important that this direct oxidative pathway be understood and, if possible, controlled. A practical method of producing L-tartaric acid by microbial oxidation of glucose would be of benefit to the food industry. There have also been several uses suggested for 2-ketogluconic acid and 5-ketogluconic acid (Stubbs et al. 1940).

The two reductases of this pathway have been partially purified and characterized from Gluconobacter liquifaciens. Chiyonobu, Adachi, and Ameyama (1973) partially purified 2-ketogluconate reductase 1000-fold using ammonium sulfate fractionation and column chromatography. Polyacrylamide disc-gel electrophoresis resulted in a single enzyme

band. The molecular weight of the enzyme was reported to be 110,000 daltons but has been recently determined to be 120,000 after crystal-lization of the enzyme (Chiyonobu et al. 1975). SDS-gel electrophoresis and amino acid analysis of the enzyme indicated that it is composed of eight subunits of identical size. The pH optimum for the in vitro oxidation of gluconic acid to 2-ketogluconic acid was 10.5, and for the reduction of 2-ketogluconic acid to gluconic acid the pH optimum was 6.5. The optimum temperature for both reactions was 50 C. An inhibition study by Ameyama et al. (1974) showed that both oxidation and reduction by 2-ketogluconate reductase were inhibited by the addition of oxalate. The importance of inhibitors of this enzyme has recently become apparent with the conclusion of Chiyonobu et al. (1976) that $NADP^+$-dependent 2-ketogluconate reductase has a role in controlling the intracellular con-centration of gluconic acid.

Chiyonobu et al. (1975) have crystallized and further characterized 2-ketogluconate reductase from both genera of the acetic acid bacteria. A comparison of the enzyme from Gluconobacter liquifaciens and Acetobacter rancens showed the similar properties of molecular weight, pH stability, and thermostability. There were some catalytic properties which differed between the two genera, however. Significant differences were found in substrate specificity, inhibitor specificity, and crystalline shape.

The $NADP^+$-dependent 5-ketogluconate reductase was partially purified and characterized by Ameyama, Chiyonobu, and Adachi (1974). A purifica-tion of 120-fold was accomplished using the same procedure for purifying the 2-ketogluconate reductase (Chiyonobu et al. 1973). This enzyme was found to be fairly unstable; at 5 C all activity was lost after three days. The optimum pH for the oxidation of gluconic acid was 10.0

and for the reduction of 5-ketogluconic acid to gluconic acid 7.5.
The optimum temperature for both reactions was 50 C.

Since NADP$^+$-dependent 5-ketogluconate reductase has not been
adequately studied, the present study was undertaken to further
characterize the NADP$^+$-dependent 5-ketogluconate reductase of
Gluconobacter suboxydans.

MATERIALS AND METHODS

Chemicals

Ammonium persulfate, $NADP^+$, phenazine methosulfate, nitroblue tetrazolium, Trisma base, and glycine were obtained from Sigma Chemical Company. Bromophenol blue, ethylene dinitrilotetraacetic acid disodium salt (Na_2EDTA), and boric acid were purchased from Matheson, Coleman, and Bell. Acrylamide (electrophoretic purity), N,N'-methylenebisacrylamide, and N,N,N',N'-tetramethylethylenediamine (TEMED) were obtained from Eastman Organic Chemical Company. All other compounds were of reagent grade and were purchased from various sources.

Organism

The microorganism used for this study was Gluconobacter suboxydans 621, which was obtained from the American Type Cultures Collection, Bethesda, Maryland.

Growth Conditions

Stock cultures of G. suboxydans were grown on slants which contained 2% glucose, 0.75% yeast extract, and 2% agar.

Cells were grown in media which contained 0.75% yeast extract and 2% concentrations of glucose, gluconate, glycerol, sorbitol, or mannitol as the sole carbon source. Inoculum for experimentation was prepared by inoculating 125 ml of medium in 250-ml Erlenmeyer flasks with the stock culture. The inoculum was incubated on a reciprocal shaker, stroke length 2.2 cm, at 28 C for 24 hours. One-liter Elenmeyer flasks which contained

5

300 ml of medium were inoculated with 20 ml of the inoculum. The
cultures were then incubated for 48 hours at 28 C on a reciprocal
shaker, stroke length 2.2 cm. Cells were harvested by centrifugation
at 12,000 x g at 4 C for 5 minutes with a Sorvall RC2-B centrifuge.

Preparation of Cell-free Extracts

Cells were washed in 0.1M potassium phosphate buffer (pH 8.5)
and suspended in the buffer. They were sonicated for 4 to 8 minutes
at 4 C with a Branson Sonifier Cell Disruptor, Model W140D (Heat System-
Ultrasonics, Inc.). Cell debris was removed by centrifugation at
37,000 x g at 4 C for 20 minutes with a Sorvall RC2-B centrifuge.

Enzyme Assay

5-Ketogluconate reductase activity was assayed by following the
reduction of $NADP^+$ at 340 nm at 25 C on a Bausch and Lomb Spectronic
505 Recording Spectrophotometer. The reaction mixture contained 70 mM
potassium phosphate buffer at pH 8.5, 3.2 mM $NADP^+$, water, and enzyme
extract in a total volume of one ml. The reaction started with the
addition of 50 mM sodium gluconate in 0.05 ml. One unit of enzyme
activity was defined as that amount of enzyme causing the reduction of
one micromole of $NADP^+$ per minute.

Polyacrylamide Disc-Gel Electrophoresis

A stock mixture of 100 g of acrylamide and 3.67 g of N,N'-
methylenebisacrylamide was used in the preparation of all gels. From
this stock mixture, 7% gels were polymerized in glass tubes (0.6 x 9.6
cm or 0.8 x 6.8 cm) using ammonium persulfate (1.5 mg/ml) as the
catalyst. Electrophoresis was carried out in a Canalco Analytical Disc-
Gel Electrophoresis Model 6 apparatus.

Two buffer systems were used for electrophoresis: a) a continuous system using TRIS-Na$_2$EDTA-borate and b) a discontinuous system using TRIS-glycine as the electrode buffer and TRIS-HCl as the gel buffer. The TRIS-Na$_2$EDTA-borate gel buffer contained 33 mM TRIS, 26.9 mM Na$_2$EDTA, and 0.03% TEMED; the electrode buffer contained 82.5 mM TRIS and 2.7 mM Na$_2$EDTA adjusted to pH 8.5 with a saturated solution of boric acid. The TRIS-HCl gel buffer was composed of 48 mM HCl, 4.4 mM TRIS, and 0.023% TEMED; the TRIS-glycine electrode buffer contained 5 mM TRIS and 4 mM glycine with a pH of 8.5.

Samples of crude enzyme extracts which contained from 0.15 to 0.25 units of 5-ketogluconate reductase per ml were adjusted to 0.05 ml with electrode buffer and placed on the gels. A saturated sucrose solution was made at 25 C and one drop was mixed with each sample to increase the density of the sample. Electrophoresis was carried out at a constant current of 5 mA per gel column using bromophenol blue as the tracking dye.

The gels were stained specifically for NADP$^+$-dependent 5-ketogluconate reductase in a reaction mixture which contained 100 mM potassium phosphate buffer at pH 8.5, 0.61 mM NADP$^+$, 0.61 mM phenazine methosulfate, 1.6 mM nitro blue tetrazolium, and 20 mM sodium gluconate, glycerol, mannitol, or sorbitol.

Anion-Exchange Chromatography

The anion-exchange columns were composed of 2.0 g of DEAE Sephadex A-50-120 swollen in 0.1 M potassium phosphate buffer at pH 7.5, and the gels were packed in 2 x 28 cm columns (Pharmacia Fine Chemicals, Uppsala, Sweden). Enzyme extract was added to the column. Two elution procedures were used: a) equilibration with 100 ml of 0.1 M potassium phosphate

buffer pH 7.5 and stepwise elution using NaCl and b) equilibration with 100 ml of 0.1 M potassium phosphate buffer and linear gradient elution. In the stepwise-elution procedure, 0.1 M, 0.2 M, and 0.3 M NaCl solutions were prepared using 0.1 M potassium phosphate buffer at pH 7.5. Fractions were collected in 7.3 ml quantities. The linear gradient elution began with a 0.1 M NaCl solution and continued to 0.4 M NaCl. Fractions were collected in 5.0 ml samples.

Gel Filtration

The Sephadex G-100 was swollen in 0.1 M potassium phosphate buffer at pH 7.5. The gel was packed in a column (3 x 46 cm) and equilibrated with 0.1 M potassium phosphate buffer pH 7.5. Crude enzyme extract was concentrated using an Amicon Ultrafiltration Cell, Model 52, with a pore size of 30,000 and a Schleicher and Schuell collodion bag, No. 100, with a pore size of 25,000 daltons. The concentrated extract was mixed with Blue Dextran (M.W. 2,000,000) and placed on the column. The extract was eluted from the gel with 0.1 M potassium phosphate buffer pH 7.5. Fractions of 6.0 ml were collected.

RESULTS

Isozymes

Crude enzyme extracts from cells grown in media which contained a 2% concentration of gluconate, glucose, glycerol, sorbitol, or mannitol as the sole carbon source were electrophoresed using a modified form of the Davis technique (1971). Prior to electrophoresis, each extract was dialyzed against the electrode buffer for one-half hour. Enzyme extracts were electrophoresed in polyacrylamide gels (0.6 x 9.6 cm) with the TRIS-Na_2EDTA-borate continuous system. Gels were incubated in reaction mixtures which contained sodium gluconate, glycerol, sorbitol, or mannitol as the substrate. Isozymes from cells grown on each medium and incubated in the reaction mixture which contained sodium gluconate had three Rf values which were comparable to each other (Figure 1). Isozymes from cells grown in media which contained glucose, gluconate, or glycerol when incubated in a glycerol reaction mixture exhibited Rf values which were comparable to two of the Rf values of isozymes obtained in the sodium gluconate reaction mixture (Table 1, Figure 2). Multiple banding patterns were obtained from extracts stained in reaction mixtures which contained sorbitol or mannitol. It was determined that the sorbitol and mannitol used in this study contained trace amounts of several alcohols which were interfering with the results. Further purification was deemed impractical, and they were excluded from the remainder of the study.

Crude enzyme extracts from cells grown in media which contained gluconate, glucose, or glycerol were electrophoresed in polyacrylamide gels (0.8 x 6.8 cm) using discontinuous electrophoresis. The electrode

9

Figure 1.   Isozymes of 5-ketogluconate reductase of <u>Gluconobacter</u>
<u>suboxydans</u> grown in media which contained glucose,
gluconate, glycerol, sorbitol, or mannitol and stained
in a sodium gluconate reaction mixture.
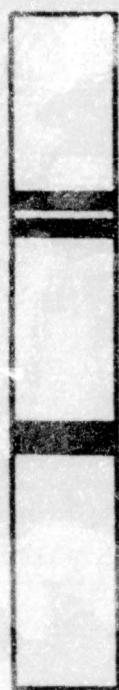
GLUCOSE SODIUM GLYCEROL SORBITOL MANNITOL

GLUCONATE

Table 1. Electrophoretic mobilities of 5-ketogluconate reductase
isozymes from Gluconobacter suboxydans grown in glucose,
gluconate, or glycerol and stained in a reaction mixture
which contained gluconate or glycerol.

| REACTION MIXTURE SUBSTRATE | MOBILITY | | |
| | CARBON SOURCE | | |
	GLUCOSE	SODIUM GLUCONATE	GLYCEROL
SODIUM GLUCONATE	0.28	0.28	0.30
	0.31	0.31	0.33
	0.63	0.65	0.66
GLYCEROL	0.27	0.28	0.29
		0.33	0.32
		0.36	

Figure 2. Isozymes of 5-ketogluconate reductase of glucose-,
gluconate-, or glycerol-grown Gluconobacter suboxydans.
Cell-free enzyme extracts were electrophoresed and
stained in reaction mixtures which contained sodium
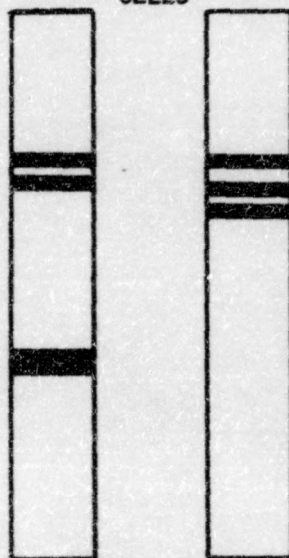gluconate (NaGlu) or glycerol (Gly).
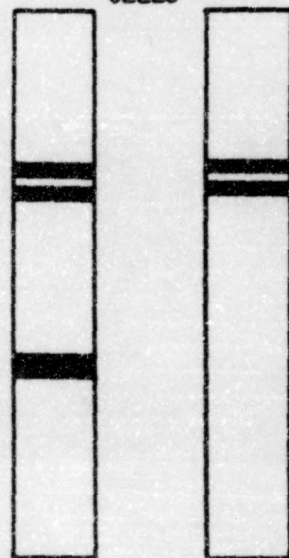
GLUCOSE-GROWN CELLS   SODIUM GLUCONATE-GROWN CELLS   GLYCEROL-GROWN CELLS

NaGlu   Gly   NaGlu   Gly   NaGlu   Gly

buffer was TRIS-glycine, and TRIS-HCl was the gel buffer. Gels were stained in reaction mixtures which contained gluconate or glycerol. The results obtained from the gluconate reaction mixture are presented in Figure 3 and Table 2. Inconclusive results were obtained from the glycerol reaction mixture. It is possible that residual electrode buffer in the gels interfered with the formation of the formazan precipitate. Isozymes present in the extracts incubated in the gluconate reaction mixture had three Rf values which corresponded to the results obtained from continuous electrophoresis (Table 1).

Separation of Isozymes

Stepwise anion-exchange chromatography

Crude enzyme extracts from cells grown in media which contained glucose or gluconate were concentrated by ultrafiltration. The extract from the glucose-grown cells contained 26 units in a volume of 13 ml. The extract from the gluconate-grown cells contained 12 units in 14 ml. The extracts were then fractionated on a DEAE Sephadex column (2 x 28 cm) by a stepwise elution procedure. Fractions were collected in 7.3 ml volumes and assayed for enzyme activity. Figures 4 and 5 represent the elution profiles obtained from the two fractionations.

Three peaks from each fractionation were resolved and designated Peaks I, II, and III. The fraction from each peak having the highest activity was subjected to disc-gel electrophoresis. The samples were electrophoresed in polyacrylamide gels (0.8 x 6.8 cm) with a TRIS-Na$_2$EDTA-borate continuous system. Gels were incubated in reaction mixtures which contained gluconate or glycerol as substrate. The Rf values obtained are presented in Table 3. Peak I of both elution

Figure 3. Isozymes present in cell-free extracts of glucose-,
sodium gluconate (NaGlu)-, or glycerol-grown
Gluconobacter suboxydans when stained in a reaction
mixture which contained sodium gluconate.

GLUCOSE
GROWN
CELLS

NAGLU
GROWN
CELLS

GLYCEROL
GROWN
CELLS
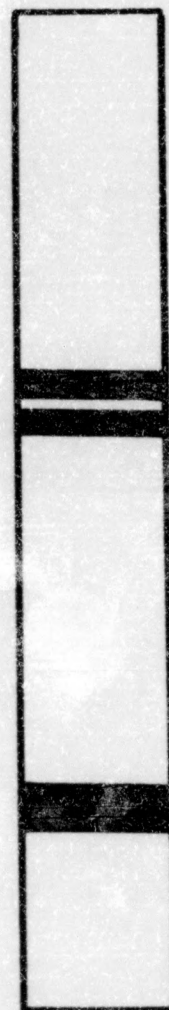
Table 2. Electrophoretic mobilities of 5-ketogluconate reductase
isozymes from Gluconobacter suboxydans grown in glucose,
gluconate, or glycerol and stained in a reaction mixture
which contained gluconate.

REACTION MIXTURE SUBSTRATE	MOBILITY		
	CARBON SOURCE		
	GLUCOSE	SODIUM GLUCONATE	GLYCEROL
SODIUM GLUCONATE	0.35	0.35	0.38
	0.39	0.39	0.41
	0.78	0.80	0.81

Table 3. Electrophoretic mobilities of 5-ketogluconate reductase isozymes contained in Peaks I,
II, and III obtained from fractionation on a DEAE Sephadex column and stained in reaction
mixtures which contained gluconate or glycerol as substrate.

REACTION MIXTURE	MOBILITY					
	PEAKS FROM GLUCOSE-GROWN CELLS			PEAKS FROM GLUCONATE-GROWN CELLS		
SUBSTRATE	I	II	III	I	II	III
SODIUM GLUCONATE	0.26	0.76	0.78	0.36	0.84	0.84
	0.37			0.41		
GLYCEROL	0.26	--	--	0.38	--	--
	0.37			0.44		

profiles (Figures 4 and 5) contained the two isozymes with the least electrophoretic mobility (Table 1, Figure 2). In addition, Peaks II and III had one band which corresponded to the isozyme with the greatest electrophoretic mobility (Table 1, Figure 2) when stained in the reaction mixture which contained gluconate. The gels from Peaks II and III which were placed in the glycerol reaction mixture had no bands.

Linear gradient anion-exchange chromatography

A crude enzyme extract from glucose-grown cells was concentrated to 3.0 ml which contained 18 units and fractionated on a Sephadex G-100 column (3 x 46 cm) as previously described. Fractions were collected in 6.0 ml volumes and assayed for enzyme activity. The elution profile of this fractionation is presented in Figure 6. Peak fractions which contained the enzyme were concentrated by ultrafiltration to 10 ml and applied to a DEAE Sephadex column (2 x 28 cm). The isozymes were eluted from the column by a linear gradient procedure. Five milliliter samples were collected. These samples were assayed for enzyme activity, and two major peaks of activity were observed (Figure 7), one of which corresponded to Peak I and one of which corresponded to Peaks II and III obtained from the stepwise elution procedure (Figure 4).

A concentrated enzyme extract (20 units in 11 ml of extract) from glucose-grown cells was also fractionated on DEAE Sephadex employing linear gradient elution without previous fractionation of Sephadex G-100. Fractions were collected in 5.0 ml volumes and assayed for enzyme activity. Again, two major peaks of enzyme activity were found (Figure 8) which corresponded to those found in the previous fractionation (Figure 7).

Figure 4. Elution profile of enzyme extract of glucose-grown
Gluconobacter suboxydans fractionated on DEAE
Sephadex G-50 by stepwise elution procedure; 7.3
ml fractions were collected.

Figure 5. Elution profile of enzyme extract of gluconate-grown
Gluconobacter suboxydans fractionated on DEAE Sephadex
G-50 by stepwise elution procedure; 7.3 ml fractions
were collected. Elution proceeded from buffer to 0.1 M
NaCl through 0.4 M NaCl.

Figure 6. Elution profile of enzyme extract of glucose-grown
Gluconobacter suboxydans fractionated on Sephadex G-100;
6.0 ml fractions were collected.

Figure 7. Elution profile of enzyme extract of glucose-grown
Gluconobacter suboxydans previously fractionated
on Sephadex G-100 and eluted by a linear gradient
procedure on DEAE Sephadex. Elution proceeded from
buffer to 0.1 M NaCl through 0.4 M NaCl. Fractions
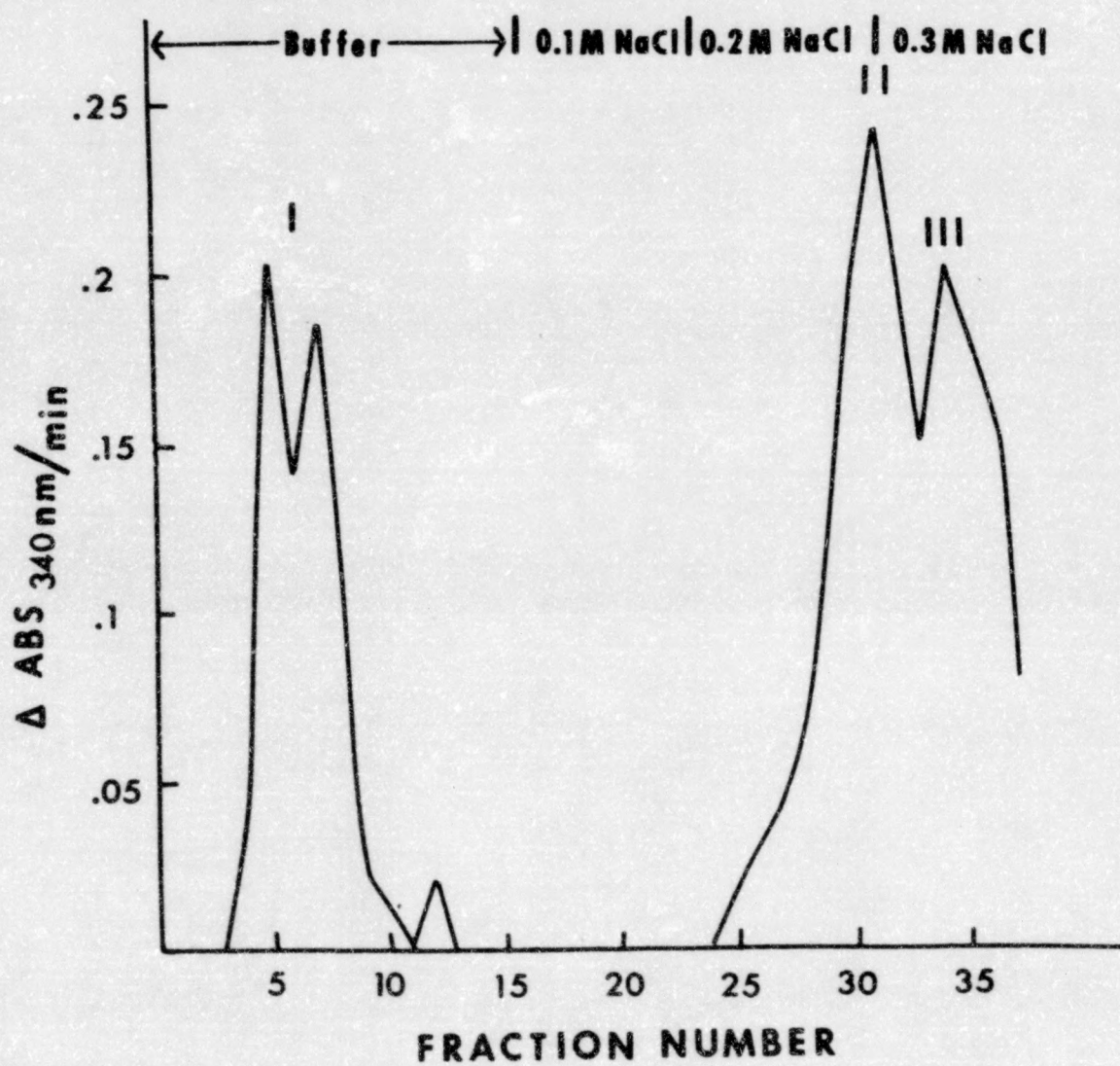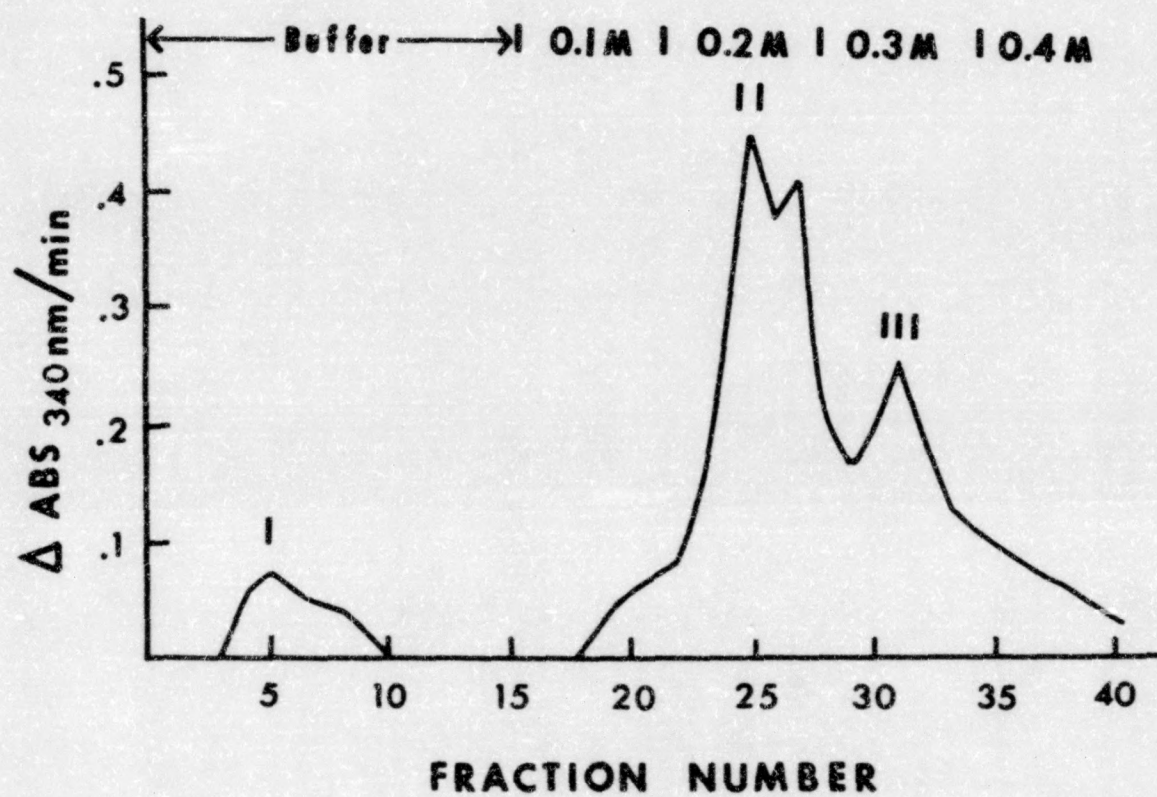were collected in 5.0 ml volumes.

Figure 8. Elution profile of enzyme extract of glucose-grown
           Gluconobacter suboxydans fractionated on DEAE
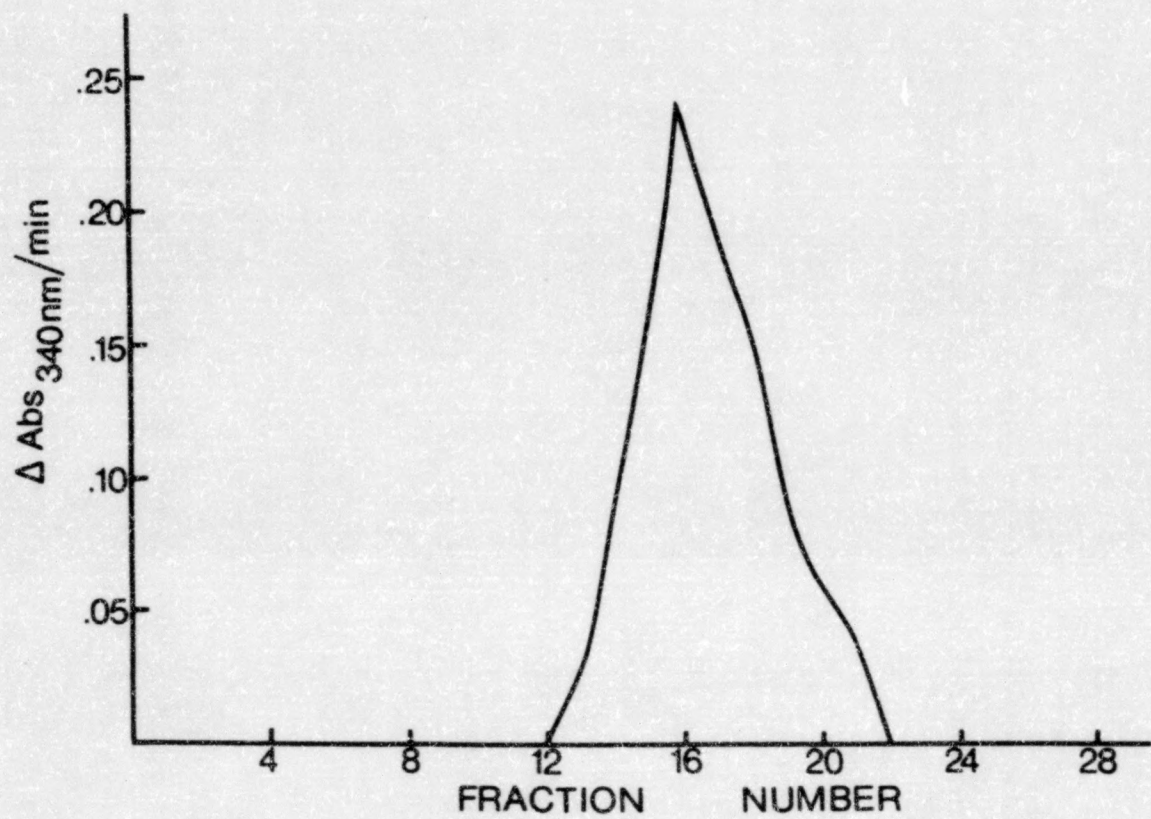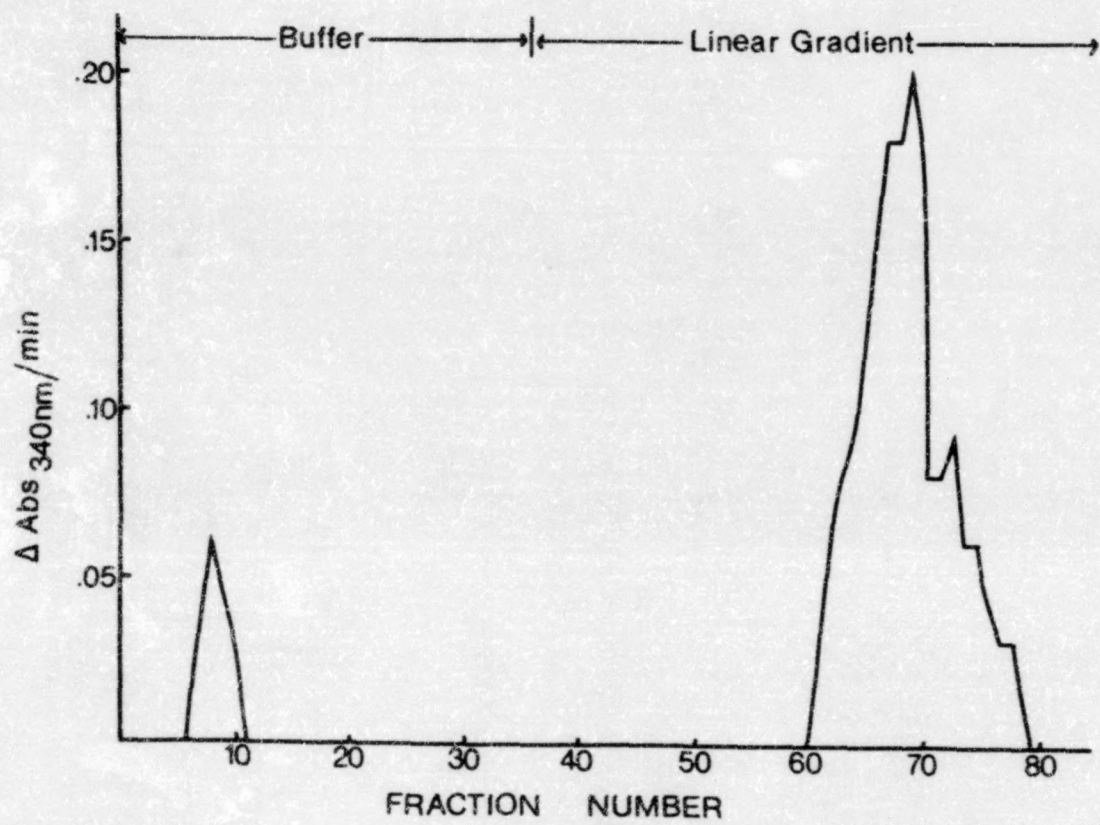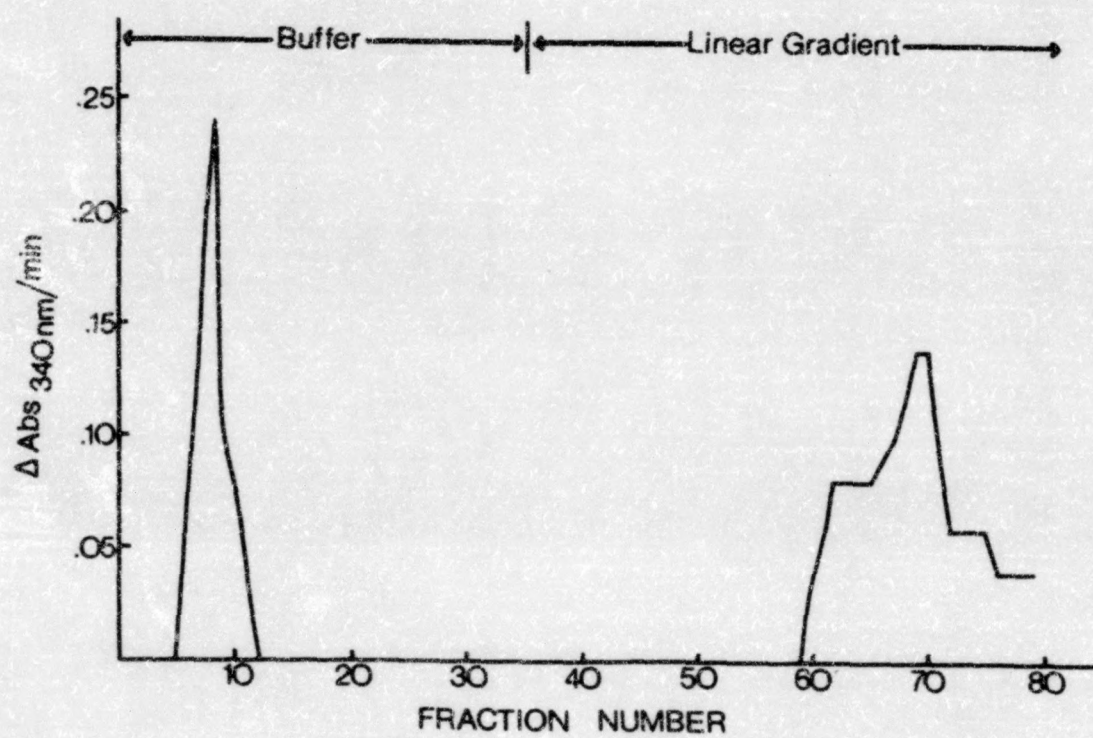   Sephadex; 5.0 ml samples were collected. Elution
           proceeded from buffer to 0.1 M NaCl through 0.4 M
           NaCl.

DISCUSSION

Polyacrylamide disc-gel electrophoresis of crude enzyme extracts
from Gluconobacter suboxydans grown in media which contained one of
five different carbon sources indicated three isozymes of 5-ketoglu-
conate reductase. Isozymes are forms of an enzyme catalyzing the
same reaction but differing in molecular weight, kinetic properties,
and electrophoretic mobilities (Self and Weitzman, 1970). Isozymes
probably are involved in regulation of metabolism and adaptation at the
cellular level. The presence of these three isozymes in G. suboxydans
may contribute to the continuation of non-phosphorylative oxidation
of glucose under varied environmental conditions.

De Ley (1958) found 5-ketogluconate reductase to be inducible in
Klebsiella and Escherichia. Evidence obtained in this study indicated
that the enzyme is present and active in cells of G. suboxydans grown
in media which contained one of five different carbon sources. It is
possible that these isozymes may catalyze the metabolism of all these
substrates in accordance with Bertrand's rule. Bertrand's rule, as
presented in the McGraw-Hill Encyclopedia of Science and Technology
(1971), can be used to predict which compounds would be dehydrogenated
by bacteria and which would not. Only those compounds which have cis
secondary alcohol groups according to the Fischer formulae with at
least one carbon of D configuration subtended by a primary alcohol group
may have the D carbon atom dehydrogenated. The substrates utilized in
this study conform to this rule, and 5-ketogluconate reductase is known
to catalyze the conversion of gluconic acid to 5-ketogluconic acid;

23

therefore, it is possible that sorbitol and mannitol are also dehy-
drogenated by the 5-ketogluconate reductase.

Enzyme stability was affected by the buffering system used
during electrophoresis. Electrophoresis using a continuous system
with 0.04 M Histidine-NaOH buffer gave inconclusive results. Enzyme
assays comparing activity in this buffer with activity in the two
buffering systems subsequently used showed a considerable decrease
in activity with the Histidine-NaOH buffer. 5-Ketogluconate reductase
has been demonstrated to be thermolabile (Ameyama et al. 1974).
Enzyme extracts for this study were kept frozen until used, and during
experimentation they were kept refrigerated (8 C) or in ice. Enzyme
activity decreased through each experiment, and after 36 hours at 8 C
only a small portion of the original activity could be detected. This
affected the results of the chromatography fractionation which required
24 hours of preparation and 12 hours for experimentation.

Stepwise anion-exchange chromatography of extracts from cells
grown in media which contained glucose or gluconate resulted in the
separation of one of the isozymes from the other two (Figure 4). Results
from this procedure also indicated that the isozyme with the greatest
electrophoretic mobility could, in fact, be two isozymes with the same
electrophoretic mobility. Samples from Peaks II and III of the elution
profile were electrophoresed and gave the same banding pattern. Linear
gradient anion-exchange chromatography was performed on enzyme extract
from glucose-grown cells to determine if Peaks II and III actually did
correspond to two overlapping isozymes. The presence of a single major
peak during this procedure where Peaks II and III would be expected
indicated that there was only one isozyme involved. The formation of

two overlapping peaks during stepwise elution on DEAE Sephadex G-50

gels may have been due to the harshness of the changes in the gradient.

Separation of the two isozymes contained in Peak I of the elution pro-

files will entail a different procedure, possibly cation-exchange

chromatography. Anion-exchange chromatography will be useful in the

separation and future purification of the third isozyme.

The formation of one peak during gel filtration of the crude

enzyme extract from glucose-grown cells indicated that all three isozymes

may have a similar molecular weight. A comparison of extracts which had

and those extracts which had not been fractionated on a Sephadex G-100

gel prior to linear-gradient DEAE Sephadex G-50 fractionation, showed

no significant differences in the elution profiles. Therefore, it is

not necessary to fractionate by Sephadex G-100 filtration before anion-

exchange chromatography. This would result in further loss of enzyme

activity during filtration, and the results do not warrant it.

The detection and partial separation of the three isozymes of

$NADP^+$-dependent 5-ketogluconate reductase from Gluconobacter suboxydans

is a significant advance in the characterization of this enzyme. The

results obtained in this study may be used in the future purification

of one of the isozymes.

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