Distribution and Solubilization of Particulate Gluconate Dehydrogenase and Particulate 2-Ketogluconate Dehydrogenase in Acetic Acid Bacteria

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The distribution of two particulate enzymes, gluconate dehydrogenase (GDH) and 2-ketogluconate dehydrogenase (2KGDH), was investigated with cell free extract through 26 strains of genus *Acetobacter* and genus *Gluconobacter*. GDH activity was found in the cell free extracts from all strains of genus *Gluconobacter* and two species of genus *Acetobacter*, *A. aceti* and *A. aurantium*. High activity of 2KGDH was also found in the pigment-producing strains of genus *Gluconobacter*.

Best solubilization of particulate enzymes was attained with the highest recovery when 10 mg of Triton X-100 and 30 mg of protein of particulate fractions in 1 ml of 0.01 M phosphate buffer, pH 6.0, are incubated for 9 hr at 5°C with continuous stirring.

By comparison of the total enzyme activity of particulate enzymes with that of NAD(P)-linked enzymes in the cell free extract, it was obvious that the formation of ketogluconates by particulate enzymes was much more predominant, roughly over 100 times higher, as that of NAD(P)-linked enzymes.

It has been well known that the growing cells or resting cells of acetic acid bacteria belonging to *Suboxydans* or *Mesoxydans* group have a marked biological activity to oxidize gluconate (GA) to 2-ketogluconate (2KGA) and 5-ketogluconate (5KGA). De Ley and Stouthamer¹⁾ have suggested that GA was oxidized by three different enzymes, two of which are NAD(P)-linked dehydrogenases yielding 2KGA or 5KGA, and particulate oxidase yielding 2KGA, in the nonphosphorylative ketogenic metabolism of the acetic acid bacteria. Our investigations have been focused on systematic understandings of these nonphosphorylative metabolic pathways of carbohydrates in the acetic acid bacteria and other oxidative bacteria. We have reported on the purification and characterization of NAD(P)-linked 2-ketogluconate reductase^{2~4)} and 5-ketogluconate reductase⁵⁾ from *Gluconobacter liquefaciens*. More recently, we have crystallized the 2-ketogluconate reductase for the first time from *G. liquefaciens*^{6,7)} and *Acetobacter*

rancens.^{7,8)}

We have also found the occurrence of two coenzyme independent dehydrogenases, gluconate dehydrogenase (GDH) yielding 2KGA and 2-ketogluconate dehydrogenase (2KGDH) yielding 2,5-diketogluconate, in the particulate fractions of *G. liquefaciens*. These particulate dehydrogenases have been little investigated, although Katznelson *et al.*^{9,10)} reported that the latter enzyme from *G. melanogenus* dehydrogenated 2KGA to 2,5-diketogluconate in the absence of NAD(P). Moreover, it was also reported that 2KGDH (EC 1. 1. 99. 4) was partially purified about 40-fold from the particulate fraction of the same strain.¹⁰⁾ However, characterization and physiological roles of these particulate enzymes in the cells of acetic acid bacteria have been little investigated up to today. We believe that it has an immediate importance to characterize in more details these metabolic pathways of the oxidative bacteria.

In this paper, therefore, the authors wish to

deal with the distribution and solubilization of particulate enzymes of GDH and 2KGDH in the species belonging to the genus *Acetobacter* and genus *Gluconobacter*.

MATERIALS AND METHODS

Chemicals. All the chemicals used in this work were commercial products. Yeast extract and pyridine coenzymes were kindly supplied from the Oriental Yeast Co., Ltd. Sodium gluconate for culture medium was a kind gift from the Nagase Co., Ltd. and the Fujisawa Pharmaceutical Ind. Co., Ltd. DEAE-Cellulose was generously supplied from the Midorijyujii Co., Ltd. Hydroxylapatite was prepared according to the method of Tiselius *et al.*¹¹⁾

Microorganisms. The microbial strains used in this work were supplied from the Institute for Fermentation, Osaka (IFO). All the strains used are listed in Table I.

Medium and cultivation. The medium for culture consisted of 20 g of Na-gluconate, 5 g of glucose, 3 g of glycerol, 5 g of polypeptone, 2 g of yeast extract, and 200 ml of potato extract¹²⁾ in 1 liter of tap water. The pH of the medium was spontaneously settled on pH 6.5 when all of these constituents were present.

Various strains of the acetic acid bacteria were transferred from the potato-glycerol agar slant¹²⁾ to 100 ml of the medium in 500 ml shaking flasks and the cultivation was carried out at 30°C for 24 hr with reciprocal shaking.

Preparation of cell free extract. The cells in the cultured broth were harvested by continuous flow centrifugation and washed with 0.01 M potassium phosphate buffer, pH 6.0. The washed cells were suspended in appropriate volumes of the buffer containing 1 mM 2-mercaptoethanol. The cell suspension was treated with 20 kHz Tomi-Seiko ultrasonic oscillator for 20 min with an exception of *G. dioxyaceticus* which was disrupted in a Vibrogen Cell Mill from the Edmund Buhler for 15 min using 0.3 mm glass beads. The cell debris was removed by centrifugation at $12,000 \times g$ for 20 min.

Fractionation of cell free extract. Cell free extract was centrifuged at $184,000 \times g$ for 60 min by a Hitachi model 55 P ultracentrifuge. Precipitate was dispersed in a small amount of the buffer and centrifuged again as above. The clear supernatant solutions were combined together and designated as a "soluble fraction" and the resultant precipitate was subjected to Triton X-100 treatment to solubilize the enzyme. Triton X-100 was added to a final concentration of 1 per cent

to the suspension of the precipitate. The mixture was stirred gently for 9 hr at 5°C and then centrifuged at $184,000 \times g$ for 60 min. The supernatant solution was designated as a "particulate fraction."

Solubilization. Studies for the best conditions of solubilization of particulate enzymes were carried out at 0~5°C, unless otherwise stated. As both particulate enzyme activities of GDH and 2KGDH were never found in the soluble fractions, the following simplified procedures were mainly used for convenience. The cell free extract was fractionated with ammonium sulfate and the fraction from 0 to 30 per cent saturation of the salt was collected and pooled in a cold room. The precipitate at this stage usually contained all of the particulate enzyme activities. The ammonium sulfate fraction was dissolved in 0.01 M buffer, pH 6.0, and dialyzed against the same buffer for 16 hr. The dialyzed solution was centrifuged at $12,000 \times g$ for 20 min and the supernatant solution (equivalent to 30 mg protein/ml) was incubated with surface active agents under various conditions. After incubation, the mixture was centrifuged at $184,000 \times g$ for 60 min to remove insoluble materials and the supernatant solution was used as enzyme solution.

Purification of particulate enzymes. GDH and 2KGDH from the cell free extract of *G. liquefaciens* were highly purified about 160- to 240-fold by a procedure involving ammonium sulfate fractionation, solubilization by Triton X-100, and DEAE-cellulose, and hydroxylapatite-column chromatographies. The solubilized enzyme was chromatographed on a DEAE-cellulose column which had been equilibrated with 0.01 M phosphate buffer, pH 6.0, containing 0.1 per cent Triton X-100. Active fraction of GDH was passed through the DEAE-cellulose column under these chromatographic conditions, and 2KGDH was eluted from the column with 0.2 M phosphate buffer, pH 6.0. GDH and 2KGDH solutions were applied to a hydroxylapatite column separately which had been equilibrated with 0.01 M phosphate buffer, pH 6.0. The columns were washed with excess amounts of 0.01 M phosphate buffer, pH 6.0, to remove Triton X-100. The enzyme eluted under a linear gradient elution made by 0.01 M and 0.4 M of the buffer. The active fractions of GDH or 2KGDH were eluted at about 0.2 M of the buffer. Specific activity of GDH and 2KGDH thus obtained was 3770 and 5660, respectively.

Enzyme assays. GDH and 2KGDH activities of particulate fractions were determined spectrophotometrically by measuring the decrease of absorbancy of 2,6-dichlorophenolindophenol at 600 nm. The reaction mixture contained 1 μ mole of 2,6-dichlorophenolindophenol, 0.33 μ mole of phenazine methosulfate, 100 μ moles of substrate (GA or 2KGA), 200 μ moles

of potassium phosphate buffer, pH 6.0, and the enzyme solution in a total volume of 3.0 ml. The enzyme reaction was carried out in a Hitachi model 124 spectrophotometer attached with a recorder at 25°C. The molecular extinction coefficient of 2,6-dichlorophenol-indophenol of $14,000 \text{ M}^{-1}$ was cited for the calculation of enzyme unit.¹³⁾ One unit of the enzyme activity was defined as the amount of enzyme catalyzing a change of 1 μmole of 2,6-dichlorophenolindophenol per minute under the standard assay conditions. NAD(P)-linked enzymes were assayed as mentioned before,^{6,13)} and one unit of this assay was defined as the amount of enzyme catalyzing a change of 1 μmole of NAD(P)H per minute.

Protein determination. Protein content was determined by the methods by Lowry *et al.*,¹⁴⁾ using bovine serum albumin as the standard.

Electrophoresis. Disc gel electrophoresis was performed under essentially the same conditions described

by Davis¹⁵⁾ using a 7.5 per cent polyacrylamide gel and Tris-glycine buffer, pH 8.3.

Location of particulate enzyme was detected by staining the gel in the standard medium containing 0.1 M substrate, 0.4 mg/ml nitro blue tetrazolium, 0.14 mg/ml phenazine methosulfate and 0.03 M potassium phosphate buffer, pH 6.0. After staining for 5 to 10 min at 25°C in the dark, colorization was stopped by transferring the gel into 7.0 per cent acetic acid. The staining intensity and location of each band were determined by tracing densitometric scanning in Toyo Densitrol DMU-33C.

Paper chromatography of reaction products. Paper chromatography was performed by the method described in the previous paper³⁾ except that the incubation system contained 0.33 μmole of phenazine methosulfate, 100 μmoles of substrate, 100 μmoles of potassium phosphate buffer, pH 6.0, and enzyme solution in a total volume of 2.0 ml.

TABLE I. DISTRIBUTION OF PARTICULATE DEHYDROGENASES IN THE CELL FREE EXTRACT AND PARTICULATE FRACTION FROM ACETIC ACID BACTERIA
The data are expressed as a specific activity.

Strain	Cell free extract		Particulate fraction	
	GDH	2KGDH	GDH	2KGDH
<i>Acetobacter</i>				
<i>A. aceti</i> IFO 3281	1	0	16	0
<i>A. aceti</i> IFO 3284	1	0	16	0
<i>A. ascendens</i> IFO 3299	0	0	0	0
<i>A. acetosus</i> IFO 3129	0	1	0	7
<i>A. xylinum</i> IFO 3288	0	0	0	0
<i>A. kützingianus</i> IFO 3222	0	0	0	0
<i>A. rancens</i> IFO 3298	0	0	0	0
<i>A. acetigenus</i> IFO 3277	0	0	0	0
<i>A. aurantium</i> IFO 3245 ^{a)}	9	2	57	18
<i>A. aurantium</i> IFO 3247 ^{a)}	2	0	31	0
<i>Gluconobacter</i>				
<i>G. liquefaciens</i> IFO 12388 ^{a)}	22	15	89	22
<i>G. melanogenus</i> IFO 12257 ^{a)}	37	31	429	86
<i>G. melanogenus</i> IFO 3293 ^{a)}	6	10	36	15
<i>G. sphaericus</i> IFO 12467 ^{a),19)}	55	56	189	10
<i>G. oxydans</i> IFO 3189	9	0	491	0
<i>G. oxydans</i> IFO 3287	2	0	21	0
<i>G. dioxyceticus</i> IFO 3272	27	1	386	15
<i>G. cerinus</i> IFO 3262	6	0	32	0
<i>G. cerinus</i> IFO 3265	9	0	171	0
<i>G. cerinus</i> IFO 3267	1	0	20	0
<i>G. gluconicus</i> IFO 3171	7	0	81	0
<i>G. roseus</i> IFO 3990	4	0	34	0
<i>G. albidus</i> IFO 3250	11	0	166	0
<i>G. suboxydans</i> IFO 3172	7	1	18	3
<i>G. suboxydans</i> IFO 12528	5	0	11	0
<i>G. suboxydans</i> var. α IFO 3254	2	0	11	0

^{a)} The pigment-producing strains.

RESULTS AND DISCUSSION

Distribution of particulate GDH and 2KGDH

The distribution of particulate GDH and 2-KGDH was surveyed with cell free extract of the genus *Acetobacter* and genus *Gluconobacter*. As shown in Table I, GDH activities were found in the cell free extract from all strains of genus *Gluconobacter* and two species of genus *Acetobacter*, in which *A. aceti* is in *Mesoxydans* group and *A. aurantium* is classified as an intermediate type strain of acetic acid bacteria.^{16,17)} High activities of particulate 2KGDH were also found in the pigment-producing strains of *Gluconobacter* listed in Table I.

The results thus obtained with cell free extract were further ascertained with soluble and particulate fractions in more details. Both particulate enzyme activities of GDH and 2KGDH did not occur in soluble fractions of the genus *Acetobacter* and genus *Gluconobacter* even in the presence of all constituents for particulate enzyme activity. As expected, all

the enzyme activities of GDH and 2KGDH predominantly occurred in the particulate fractions. The distribution of GDH and 2KGDH of the particulate fractions showed the same results as obtained with cell free extract. These results also suggest the possibility to solubilize the enzymes by a surface active agent, and the solubilization could be actually performed by Triton X-100.

Identification of reaction products produced by cell free extract

As shown in Table II, the reaction products by the particulate enzymes were examined with cell free extracts and GA or 2KGA as substrate. Although no reaction products of GA or 2KGA could be detected by the enzymes from genus *Acetobacter*, the cell free extracts from some of the species of genus *Gluconobacter*, except *G. dioxyaceticus* and two strains of *G. suboxydans* produced 2KGA, but not 5KGA from GA. However, the cell free extracts from *G. dioxyaceticus* and two strains of *G. suboxydans* produced 5KGA from GA as they are designated as 5KGA-producing

TABLE II. REACTION PRODUCTS FROM GLUCONATE OR 2-KETOGLUCONATE BY CELL FREE EXTRACT

Strain	Product		
	2-Keto-glucuronate ^{a)}	5-Keto-glucuronate ^{a)}	2,5-Diketo-glucuronate ^{b)}
<i>G. liquefaciens</i> IFO 12388 ^{c)}	+		+
<i>G. melanogenus</i> IFO 12257 ^{c)}	+		+
<i>G. melanogenus</i> IFO 3293 ^{c)}	+		+
<i>G. sphaericus</i> IFO 12467 ^{c),18)}	+		+
<i>G. oxydans</i> IFO 3189	+		
<i>G. oxydans</i> IFO 3287		+	
<i>G. dioxyaceticus</i> IFO 3272	+		
<i>G. cerinus</i> IFO 3262	+		
<i>G. cerinus</i> IFO 3265	+		
<i>G. cerinus</i> IFO 3267	+		
<i>G. gluconicus</i> IFO 3171	+		
<i>G. roseus</i> IFO 3990	+		
<i>G. albidus</i> IFO 3250	+		
<i>G. suboxydans</i> IFO 3172	+		
<i>G. suboxydans</i> IFO 12528		+	
<i>G. suboxydans</i> var. α IFO 3254		+	

a) Reaction product from gluconate as a substrate.

b) Reaction product from 2-ketogluconate as a substrate.

c) The pigment-producing strains.

Gluconobacter. This interesting finding suggests that two kinds of GDH, yielding 2KGA or 5KGA, must be present in the genus *Gluconobacter*. In addition, the cell free extract from the pigment-producing strains of *Gluconobacter* catalyzed the formation of 2,5-diketogluconate from 2KGA. These results are in fairly good agreement with the distribution of particulate enzymes shown in Table I.

Effect of various surface active agents on the solubilization of particulate enzymes

Various surface active agents were added to the particulate fractions to check the effect of solubilization of the enzymes. Incubation of particulate fractions with surface active agents was performed at 5°C for 9 hr under continuous shaking. As shown in Table III, sodium deoxycholate and Triton X-100 were found to be the best of all surface active agents tested to solubilize the particulate 2KGDH with a good

recovery. GDH was also solubilized by sodium deoxycholate, Triton X-100 and Tweens. Sodium deoxycholate, however, was found to be unsuitable for the solubilization of the enzymes, since it made the resulting enzyme solution getting viscous and some difficulty occurred in the subsequent handlings. Finally, Triton X-100 was evaluated to be the best of all for solubilization of particulate GDH and 2KGDH of the acetic acid bacteria. Thus, this agent was used throughout the following experiments.

Effects of Triton X-100 concentration, temperature and pH on solubilization of particulate enzymes

The effect of Triton X-100 concentration on enzyme solubilization was tested in 0.01 M phosphate buffer, pH 6.0, using particulate fraction from *G. liquefaciens*. As shown in Fig. 1, the best result of solubilization of the

TABLE III. EFFECT OF SURFACE ACTIVE AGENTS ON SOLUBILIZATION OF PARTICULATE ENZYMES

Reagent	Conc. (%)	Recovery (%)	
		2KGDH	GDH
Sodium deoxycholate	0.5	86	92
	1.0	4	26
Sodium stearate	0.5	0	2
	1.0	1	0
Sodium laurylsulfate	0.5	25	64
	1.0	0	0
CPC ^{a)}	0.5	3	19
	1.0	1	9
CTAB ^{b)}	0.5	0	2
	1.0	1	0
Aerosol OT	0.5	5	1
	1.0	4	3
Triton X-100	0.5	70	75
	1.0	85	86
Tween 20	0.5	26	96
	1.0	16	93
Tween 40	0.5	5	75
	1.0	4	84
Span 20	0.5	0	0
	1.0	0	0
Span 40	0.5	0	0
	1.0	0	0
Control		0	0

^{a)} Cetylpyrimidium chloride.

^{b)} Cetyltrimethyl ammonium bromide.

FIG. 1. Effect of Concentration of Triton X-100 on Solubilization of Particulate Enzymes.

The solubilization was carried out at 5°C for 9 hr in 0.01 M phosphate buffer, pH 6.0. The final concentration of protein was adjusted to 30 mg/ml. ○—○, GDH; ●—●, 2KGDH.

particulate GDH and 2KGDH was obtained at 1.0 per cent of the surface active agent. The effect of temperature on enzyme solubilization was also tested in 0.01 M phosphate buffer, pH 6.0. Incubation was carried out at 5°C, 20°C and 30°C. As shown in Fig. 2, incubation at 5°C gave a fairly good solubilization with the highest recovery of the enzyme activi-

FIG. 2. Effect of Temperature on Solubilization of Particulate Enzymes.

The solubilization of the enzymes was carried out in 0.01 M phosphate buffer, pH 6.0. One per cent of Triton X-100 and 30 mg/ml of protein were incubated for periods as indicated at different temperatures with continuous stirring.

×—×, 5°C; ○—○, 20°C; ●—●, 30°C.

FIG. 3. Effect of pH on Solubilization of Particulate Enzymes.

The solubilization of the enzymes was carried out at 5°C in 0.01 M phosphate buffer at various pH as indicated. One per cent of Triton X-100 and 30 mg/ml of protein were incubated under continuous stirring.

×—×, 3 hr; ○—○, 6 hr; ●—●, 9 hr.

ties. Incubation at high temperature should be avoided since the enzyme activity was gradually lost during the incubation.

The effect of pH on enzyme solubilization was tested in 0.01 M phosphate buffer, of which pH was varied as indicated in Fig. 3. A good solubilization and the highest recovery of GDH and 2KGDH were observed at pH 5.0 and 6.0, respectively. As mentioned above, it was concluded that the best conditions for solubilization of particulate GDH and 2KGDH could be described as follows. Ten mg of Triton X-100 and 30 mg of protein in 1 ml of 0.01 M phosphate buffer, pH 6.0, should be

incubated for 9 hr at 5°C under continuous stirring. In cases of other strains of acetic acid bacteria, the same experiments were done with the particulate fraction from individual cell free extract. The results were essentially the same with *G. liquefaciens*. Other factors that might affect the conditions of solubilization of the enzymes were also extensively tested by changing the buffer concentration, with various kinds of buffer solutions, but no additional information was obtained. It is also advantageous to perform the solubilization at weak acidic pH range since both GDH and 2KGDH have a stable pH range around 6.

Comparison between GDH and 2KGDH in electric properties

Using a highly purified enzyme preparation of GDH and 2KGDH from *G. liquefaciens*, electrophoretic analyses were carried out under the conditions described in MATERIALS AND METHODS. Electrophoretic mobilities of two particulate enzymes were different from each other and R_m values were obtained to be 0.48 for GDH and 0.40 for 2KGDH. It could be concluded that particulate GDH and 2KGDH are different species of enzymes and have each independent character in ketogenic metabolism of carbohydrates. One would participate in 2KGA production and the other would catalyze the 2,5-diketogluconate formation from 2KGA. It could be speculated that both GDH and 2KGDH are localized close to each other in subcellular localization, because either one enzyme was always accompanied by the other during the course of extraction, solubilization and purification until they were separated by ionexchange chromatography on DEAE-cellulose column (see MATERIALS AND METHODS).

Identification of reaction products by highly purified particulate enzymes

Highly purified particulate GDH or 2KGDH from *G. liquefaciens* was incubated with GA or 2KGA, respectively, in the presence of phenazine methosulfate as an electron carrier. An aliquot of the reaction mixture was spotted on a filter paper and developed as mentioned before.²¹ As shown in Fig. 4, GDH catalyzed the 2KGA formation from GA and 2KGDH catalyzed the conversion of 2KGA to 2,5-diketogluconate only in the presence of phenazine methosulfate. This result suggests that the enzyme dehydrogenating 2KGA may be the same one as described by Katznelson *et al.*^{9,10} Katznelson *et al.* reported that 2KGA was oxidized by an enzyme preparation from *G. melanogenus* and the resulting product was identified to be 2,5-diketogluconate.

FIG. 4. Paper Chromatogram of Reaction Products by Highly Purified Enzymes.

One dimensional ascending paper chromatography on Toyo Filter Paper No. 51 was employed with *tert*-butanol: formate: water (4: 1: 1.5 by volume).

Reaction products were detected by BPB, ○; BPB and TTC, ●.

1, GA; 2, 2KGA; 3, 5KGA; 4, 2,5-diketogluconate; 5, GA + GDH + phenazine methosulfate; 6, 2KGA + 2KGDH + phenazine methosulfate.

Comparison between particulate enzymes and NAD(P)-linked enzymes

As we have suggested on glucose metabolism in acetic acid bacteria in the previous papers,^{7,18} that the particulate enzyme activity has a main role in ketogluconate production, while NAD(P)-linked enzymes have another role to control the intracellular concentration of gluconate. Because, the NAD(P)-linked enzymes are essentially reversible in the reaction and the reduction of any ketogluconates to gluconate is extremely predominant around pH 6.²¹ In addition, the particulate enzymes catalyze the oxidation of GA to 2KGA or 5KGA and the oxidation of 2KGA to 2,5-diketogluconate but the reverse reaction (reduction) has never occurred by the particulate enzymes so far investigated. Both NAD(P)-linked enzymes and particulate enzymes in the cell free extract of all species of acetic acid bacteria are summarized in Table IV. In this experiment, total enzyme activity of particulate enzymes was compared with that of NAD(P)-linked enzymes in the cell free extract, using the definition of the international unit (U). It was obvious as seen in this

TABLE IV. COMPARISON BETWEEN PARTICULATE ENZYMES AND NAD(P)-LINKED ENZYMES IN THE CELL FREE EXTRACT OF ACETIC ACID BACTERIA

The expression of both enzyme activities was made using the definition of the international unit ($\mu\text{mole}/\text{min}$).

Strain	Particulate enzyme activity (units/ml)	NAD (P)-linked enzyme activity (units/ml)		
	GA to 2KGA or 5KGA	GA to 2KGA or 5KGA	2KGA to GA	5KGA to GA
<i>Acetobacter</i>				
<i>A. aceti</i> IFO 3281	14	0.45	1.26	0.61
<i>A. aceti</i> IFO 3284	36	0.10	1.77	0.35
<i>A. ascendens</i> IFO 3299	0	0	1.61	0.74
<i>A. acetosus</i> IFO 3129	0	0	0.81	0.19
<i>A. xylinum</i> IFO 3288	0	0	0.06	0.03
<i>A. kützingianus</i> IFO 3222	0	0	1.65	0.39
<i>A. rancens</i> IFO 3298	0	0.16	3.35	0.55
<i>A. acetigenus</i> IFO 3277	0	0	1.29	0.42
<i>A. aurantium</i> IFO 3245 ^{a)}	81	0.32	2.42	0.26
<i>A. aurantium</i> IFO 3247 ^{a)}	29	0.06	0.39	0.11
<i>Gluconobacter</i>				
<i>G. liquefaciens</i> IFO 12388 ^{a)}	200	0.68	1.81	0.35
<i>G. melanogenus</i> IFO 12257 ^{a)}	629	0.52	1.26	0.42
<i>G. melanogenus</i> IFO 3293 ^{a)}	71	0.29	1.87	0.16
<i>G. sphaericus</i> IFO 12467 ^{a),19)}	814	0.65	1.55	0.48
<i>G. oxydans</i> IFO 3189	150	0.77	0.50	0.81
<i>G. oxydans</i> IFO 3287	73	2.47	2.79	0.25
<i>G. dioxycetonicus</i> IFO 3272	700	0.94	1.65	1.94
<i>G. cerinus</i> IFO 3262	121	1.29	2.06	1.87
<i>G. cerinus</i> IFO 3265	150	0.61	1.55	0.97
<i>G. cerinus</i> IFO 3267	29	0.53	1.61	1.23
<i>G. gluconicus</i> IFO 3171	129	0.32	1.32	0.44
<i>G. roseus</i> IFO 3990	35	0.13	0.81	0.35
<i>G. albidus</i> IFO 3250	171	0.06	0.68	0.16
<i>G. suboxydans</i> IFO 3172	149	0.17	0.57	0.64
<i>G. suboxydans</i> IFO 12528	24	0.28	0.02	0.36
<i>G. suboxydans</i> var. α IFO 3254	43	0.84	0.48	3.42

^{a)} The pigment-producing strains.

table that the formation of ketogluconates by particulate enzymes was much more predominant, roughly over 100 times higher, than that by NAD(P)-linked enzymes. It seems likely that the NAD(P)-linked enzymes could mainly contribute to the reduction of any ketogluconates to gluconate as shown in the right frame of Table IV.

Most strains of genus *Acetobacter*, except *A. aceti* and *A. aurantium*, did not have the enzymes oxidizing GA to 2KGA or 5KGA in the presence or absence of electron carriers such as NAD (P) or dyes. High enzyme acti-

vities of particulate GDH and 2KGDH were found in the most strains of genus *Gluconobacter*, particularly in pigment-producing strains of the acetic acid bacteria. It could be concluded that the basis of classification of acetic acid bacteria into two genera, *Acetobacter* and *Gluconobacter*, is also supported by enzymatic characteristics which are intrinsic nature of the acetic acid bacteria.

The details of the particulate GDH and 2KGDH from *G. liquefaciens* are now under processing and will be reported elsewhere.

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