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NADPH-Dependent Glyoxylate Reductase in *Proteus vulgaris**

By

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It has been reported that isocitrate was cleaved to succinate and glyoxylate by isocitrate lyase (EC 4.1.3.1) (1), and α -ketoglutarate, another expectant product, was not detected by cell-free extracts of *Proteus vulgaris* under anaerobic condition (2). α -Ketoglutarate was found to be converted to succinic semialdehyde in *P. vulgaris* (3), but the semialdehyde also was not detected under the same condition. Recently, it was found that NADP was destructed by NADP-pyrophosphatase (EC 3.6.1.?) in cell-free extracts of *P. vulgaris* (4). So, the reason why α -ketoglutarate was not formed was almost clear. However, since glyoxylate is formed easily, if NADPH-dependent glyoxylate reductase is operating in this bacterium, only catalytic amount of NADPH must be sufficient for α -ketoglutarate formation. Now, it becomes an important problem whether glyoxylate reductase is present or not in *P. vulgaris*. As far as we know, there is only one brief report referring to glyoxylate reductase in this bacterium (5).

In this paper, it is reported that a NADPH-dependent glyoxylate reductase was confirmed in *P. vulgaris*.

Materials and Methods

Chemicals. NADP⁺ and NADH were purchased from Boehringer Mannheim. NADPH was obtained from Sigma Chemical Co., and Boehringer Mannheim. Sodium DL-isocitrate was a product of Sigma Chemical Co. α -Ketoglutaric acid and 2,7-dihydroxynaphthalene were the products of Tokyo Kasei Kogyo Co., Ltd. Sodium glyoxylate was obtained from Nakarai Chemicals, Ltd.

Assays. Activities of isocitrate dehydrogenase (EC 1.1.1.42) and

* Dedicated to Prof. Yositeru NAKAMURA on the occasion of his retirement.

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glyoxylate reductase were assayed by readings of increase and decrease in absorbance at 340 nm. For the assay of glyoxylate reductase, two reaction mixtures were used; System A contained in a final volume of 3.0 ml, 33.3 mM of phosphate buffer (pH 7.4), 50 μ M of NADPH, 2.5 mM of glyoxylate and appropriate amount of the enzyme, and System B was same as the former, except that 60 μ M of NADP⁺ and 1.7 mM of isocitrate were added instead of NADPH. In the latter case, glyoxylate was added after NADP⁺ was thoroughly reduced by isocitrate dehydrogenase in the glyoxylate reductase preparations, so the absolute activity of glyoxylate reductase was difficult to determine because the more NADP⁺ was formed the higher activity of isocitrate dehydrogenase had to appear.

Glyoxylate and glycollate were determined by the methods of McFADDEN and HOWES (6) and of SASAKAWA (7), respectively. Glycollate was also identified by paper chromatography using ethanol: ammonia: water (80:5:15, v/v) as a solvent system. Reaction mixture was the same as described in the assay for glyoxylate reductase, except that 27 μ M of NADP⁺ and 0.8 mM of isocitrate were used. At the indicated time of incubation at room temperature (about 20C), trichloroacetic acid was added to 5%. After deproteinization, 0.2 ml of the supernatant was subjected for the glyoxylate assay. In the case of the assay for glycollate, to overcome the interference with glyoxylate 0.5 ml of 0.2% 2,4-dinitrophenylhydrazine was added to 1.5 ml of the supernatant. The sample was kept at room temperature overnight and extracted twice with the same volume of ethylacetate. Then the aqueous layer was subjected for the glycollate assay.

Enzyme sources. Most of the experiments were carried out with sonic extracts or ammonium sulfate fraction prepared from *P. vulgaris*. *P. vulgaris* was cultivated at 37C for about 20 hr on the ordinary peptone-broth agar plates. Bacteria were harvested, washed three times with deionized water and disrupted by sonic oscillation at 20 kc for 7 min. Supernatant obtained after centrifugation at 12,000 \times g for 20 min was designated as sonic extract. Sonic extract was further treated with 1% protamine sulfate, ammonium sulfate (usually 30 to 60% saturation), dialyzed against running water for 2 or 3 hr and kept at 4C or -20C. This enzyme was designated as ammonium sulfate fraction.

Sonic extracts of *Escherichia coli* as a control organism were prepared by the same manner as *P. vulgaris*.

Results

Effect of glyoxylate on the activity of NADP-isocitrate dehydrogenase.

Reduction of NADP⁺ by isocitrate dehydrogenase was inhibited markedly with glyoxylate in *P. vulgaris*, but not in *E. coli* (Fig. 1). In *P. vulgaris*, the inhibition by glyoxylate was also observed in the cases of isovaleraldehyde and malate dehydrogenations. NADPH once formed by the oxidation of these substrates was rapidly oxidized by addition of glyoxylate, while in *E. coli* system, the oxidation of NADPH by glyoxylate did not occur (Fig. 2). When the ratio of glyoxylate to isocitrate was selected suitably, reduction and oxidation of NADP by alternate addition of isocitrate and glyoxylate could be continued several times. In the case of the inhibition by 2.5 μ moles of glyoxylate in Fig. 1, apparent reduction of NADP⁺ by isocitrate was inhibited almost completely, but during then glyoxylate was consumed as seen in Table 1.

As a matter of course, it was supposed that glyoxylate reductase in *P. vulgaris* had to couple with isocitrate dehydrogenase *via* oxidation and reduction of NADP. However, the oxidation of NADPH (Sigma, chemical

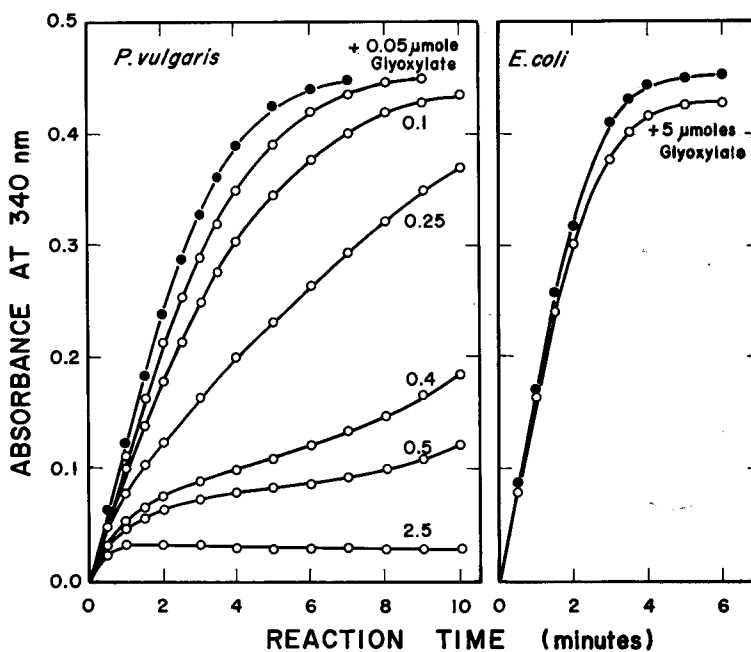


Fig. 1. Effects of different concentrations of glyoxylate on isocitrate dehydrogenase activities of *P. vulgaris* and *E. coli*. Reaction mixture was the same as described in Materials and Methods (System B), except that indicated amount of glyoxylate and sonic extract of *P. vulgaris* (0.76 mg protein) or *E. coli* (1.07 mg protein) were used.

reduced) by glyoxylate was much slower than those of NADPH reduced by substrates at an apparent ratio of 1:8 (Table 2). Then the authors presented an assumption that the glyoxylate-coupling reaction might be catalyzed by an enzyme differing from the usual glyoxylate reductase.

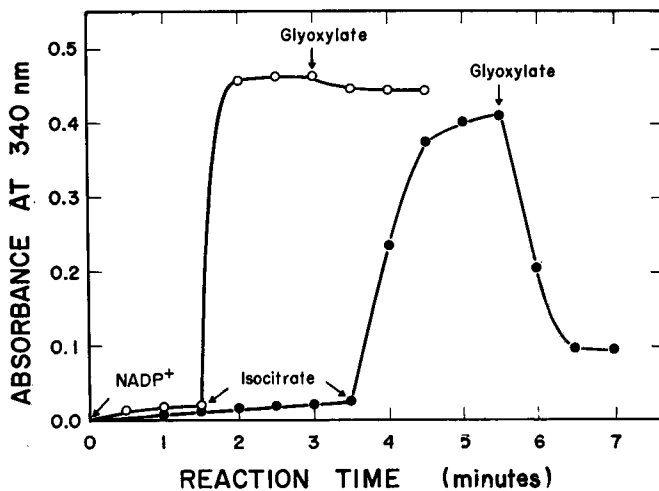


Fig. 2. Effects of glyoxylate on the NADPH reduced thoroughly by isocitrate dehydrogenase in *P. vulgaris* (●) or *E. coli* (○). Reaction mixtures were identical with those used in the experiments described in Fig. 1, except that 5 μ moles of glyoxylate was added at the indicated time.

TABLE 1. Consumption of glyoxylate during the glyoxylate-coupling reaction^a

Substrate	Glyoxylate found (μ moles/3 ml)	Glyoxylate consumed (μ moles/3 ml)
Glyoxylate	2.34	—
Isocitrate+glyoxylate	0.82	1.52
Isocitrate+glyoxylate ^b	1.50	0.82
Isocitrate	0.00	00.0

^a Reaction mixture as in Materials and Methods (System B) and in Fig. 1 (2.5 μ moles of glyoxylate). Incubation: at room temperature (about 20C) for 10 min.

^b Glyoxylate was added after 5 min of incubation.

TABLE 2. Oxidation rates of different NADPH's and NADH^a

Sort of NADPH or NADH	Oxidation rate (nmoles NAD(P)H oxidized/30 sec)	Relative rate (%)
Sigma's NADPH	10.8	9.5
Boehringer's NADPH	114.6	100.0
Substrate-reduced NADPH	89.1 ^b	78.3 ^b
Boehringer's NADH	1.14	0.1

^a Reaction mixture as System A in Materials and Methods. Ammonium sulfate fraction was used.

^b This is an apparent value because of the reason described in Materials and Methods.

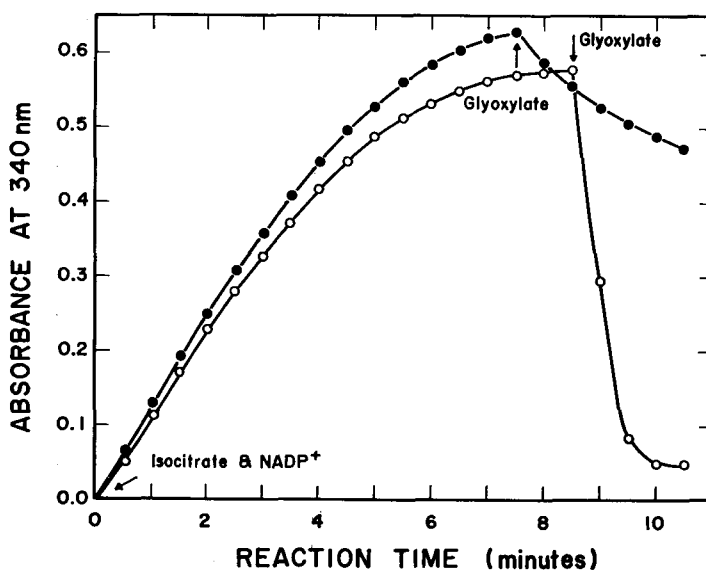


Fig. 3. Effects of glyoxylate on the substrate-reduced NADPH by sonic extract and a partially purified isocitrate dehydrogenase of *P. vulgaris*. Partially purified enzyme was prepared as follows: Frozen ammonium sulfate fraction was thawed at 30C and precipitated with chilled acetone (60%). The precipitate was dissolved in deionized water and dialyzed against running water at 12C. Reaction mixture as in Materials and Methods (System B). Symbols: ○, sonic extract; ●, partially purified isocitrate dehydrogenase.

Separation of the glyoxylate-coupling reaction from isocitrate dehydrogenase. If the glyoxylate-coupling reaction is catalyzed by a separate protein, presumably unusual glyoxylate reductase, the glyoxylate-coupling activity could be removed from the dehydrogenase by a purification of the dehydrogenase or by some specific inhibitors. As was expected, the glyoxylate-coupling activity was obviously diminished in a comparatively purified enzyme (Fig. 3). Although the data are not represented, when sonic extract was treated with Nagarse ($25 \mu\text{g}/\text{mg}$ enzyme protein) at 30°C for 90 min and with ammonium sulfate (50% saturation), the glyoxylate-coupling activity was lost almost completely.

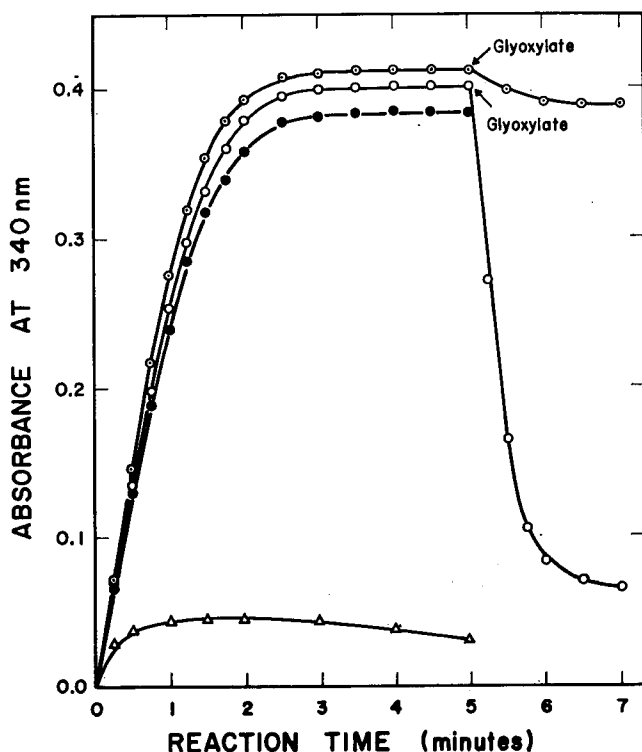


Fig. 4. Effect of an inhibitor on isocitrate dehydrogenase and glyoxylate-coupling reaction. Reaction mixture as in Materials and Methods (System B), except that 0.2 ml of ammonium sulfate fraction, 0.8 mM of isocitrate and 0.1 mg of aerated sodium hydrosulfite were used. Symbols: \triangle , isocitrate+glyoxylate+inhibitor; \bullet , isocitrate+glyoxylate+inhibitor; \circ , isocitrate+glyoxylate (added at the time indicated by the arrow); and \odot , isocitrate+inhibitor+glyoxylate (added at the time indicated by the arrow).

On the other hand, among different inhibitors tested, chlorpromazine, and p-chloromercuribenzoate were found to inhibit the activity intensely, but these inhibitors also inhibited isocitrate dehydrogenase. It was of interest that the aerated sodium hydrosulfite (Wako Pure Chemical Ind., Ltd.: chemical grade), which was incapable of acting as a reductant, did also inhibit the activity markedly, but not inhibit isocitrate dehydrogenase at all (Fig. 4), while the aged Kahlbaum's sodium hydrosulfite had no such effect. Now, it occurred to us that the weakened oxidation of Sigma's chemically reduced NADPH might be due to contaminant such as this inhibitor. So, Boehringer's enzymatically reduced NADPH was used as compared with Sigma's NADPH. The former was oxidized ten times faster than the latter as expected (Table 2).

Confirmation of glyoxylate reductase. From these above results, it was undoubted that a glyoxylate reductase was present in cell-free extracts of *P. vulgaris*. To make sure, it was attempted to recognize glycollate as a reaction product of the glyoxylate reductase. Authentic glycollate and a reaction product which could be oxidized by the glycollate oxidase (EC

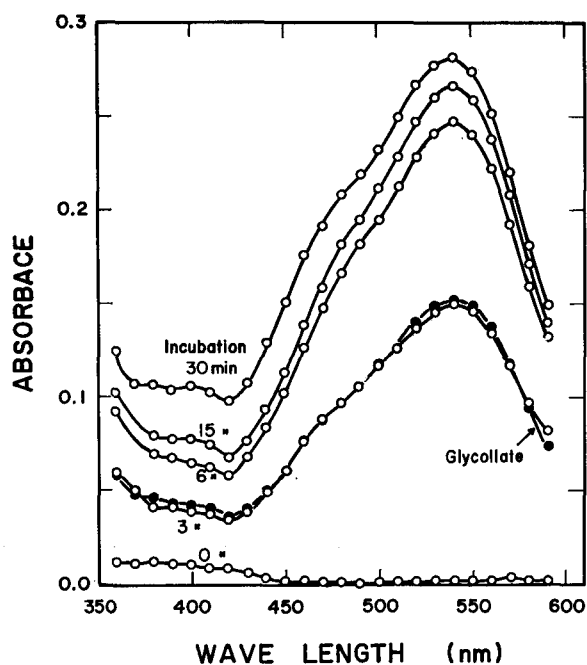


Fig. 5. Absorption spectra of glycollate and the reaction product. Conditions are described in Materials and Methods. Ammonium sulfate fraction (0.89 mg protein) was used.

1.1.3.1) prepared from spinach leaves in the preliminary experiment, showed same absorption spectra after treatment with 2,7-dihydroxynaphthalene (Fig. 5), and the same Rf values in paper chromatography at 0.49. Furthermore, as shown in Table 3, stoichiometry of the reaction supports that a glyoxylate reductase is operating in *P. vulgaris*. In that case, glycollate has to be detected as a metabolite of glucose under the same condition as previously (8). Glycollate was practically detected, but its amount was considerably small at about one per cent of added glucose, though it is

TABLE 3. Stoichiometry of the glyoxylate reductase reaction^a

Incubation time (min)	Glyoxylate consumed (μ mole)	Glycollate produced (μ mole)	Glycollate/ glyoxylate
3	0.525	0.462	0.88
6	0.873	0.759	0.87
15	1.023	0.849	0.83
30	1.095	0.891	0.81

^a Reaction mixture as System B in Materials and Method, except that 27 μ M of NADP⁺, 0.8 mM of isocitrate and 0.2 ml of ammonium sulfate fraction were used.

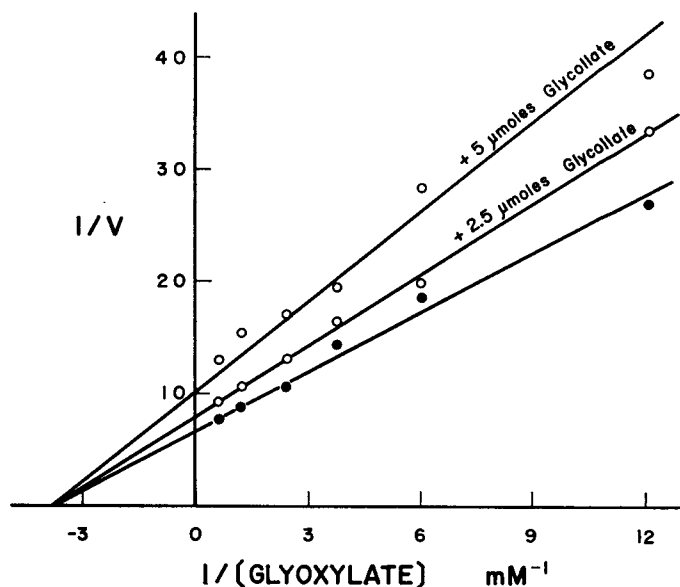


Fig. 6. Inhibition of glycollate on the glyoxylate reductase. Reaction mixture as in Materials and Methods except that 0.1 ml of ammonium sulfate fraction, 2.5 μ moles or 5 μ moles of glycollate and different amount of glyoxylate were used.

only rough estimation.

Glycollate did not react with NADP^+ in *P. vulgaris*, but glycollate inhibited the glyoxylate reductase activity non-competitively as seen in Fig. 6.

Optimal pH of the reaction was found to be about 6.8 as seen in Fig. 7, but considerably high activity was observed at a broad range of pH. K_m values for glyoxylate and NADPH were calculated to be $263 \mu\text{M}$ and $14.3 \mu\text{M}$, respectively, by Lineweaver-Burk plots (Figs. 6 and 8).

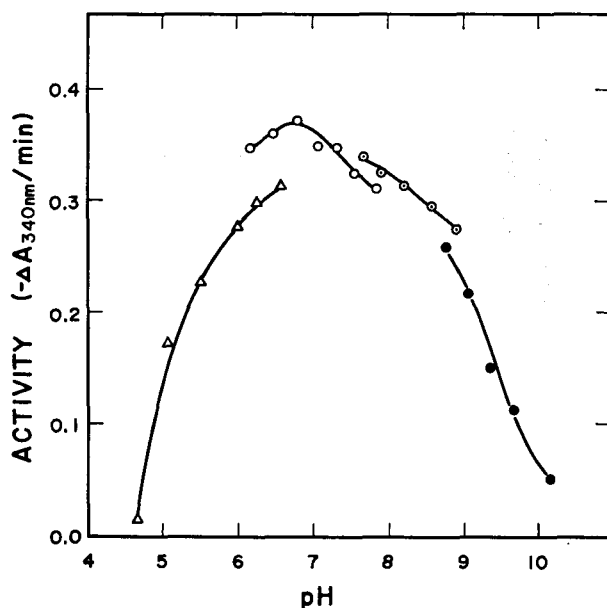


Fig. 7. Optimal pH of the glyoxylate reductase. Reaction mixture contained, in a final volume of 2.0 ml: $174 \mu\text{g}$ of ammonium sulfate fraction, $75 \mu\text{M}$ of NADPH, 2.5mM of glyoxylate and 33.3mM of phosphate buffer (O), 15mM of Tris-HCl (O with dot), 125mM of glycine-KOH (●), or citric acid- Na_2HPO_4 buffer (Δ).

Discussion

At the beginning of this study, it was supposed that the glyoxylate-coupling reaction might be catalyzed by an unusual glyoxylate reductase by the following reasons: (i) Mass spectrum of the purified reaction product was similar to that of the authentic glycollate, but did not completely agree each other; (ii) R_f values in paper chromatography of the above two samples were delicately different only in the case of the above described

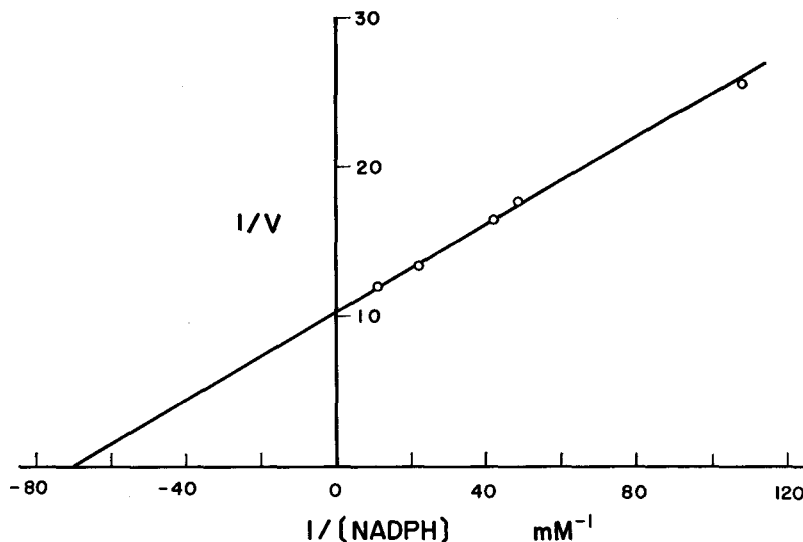


Fig. 8. K_m for NADPH. Conditions were the same as in Fig. 7, except that $37 \mu\text{g}$ of ammonium sulfate fraction, 33.3 mM of phosphate buffer (pH 7.4) and different amounts of NADPH were used.

solvent system; (iii) oxidation rate of Sigma's NADPH were remarkably slower than the glyoxylate-coupling reaction; and (iv) if glyoxylate reductase was operating in *P. vulgaris*, α -ketoglutarate and glycollate had to be detected besides succinate, glyoxylate, and lactate.

As for (i), although it was not examined repeatedly, the delicate difference may be thought to arise in consequence of a small amount of contaminant in the product preparation. Delicate difference of the Rf values (ii) was solved when the authentic sample was treated under the exactly same conditions as the reaction product. As for (iii), it could be thought that Sigma's NADPH might contain an inhibitor such as that reported in this paper, as stated in the next paragraph too. Thus, most of the foundations by which an unusual enzyme was supposed were excluded. Furthermore, from the result of the stoichiometry of the reaction it can be concluded that the usual glyoxylate reductase is present in *P. vulgaris*.

Among the inhibitors tested, an impurity in sodium hydrosulfite was found to inhibit the glyoxylate reductase specifically. Although it was not reported here in detail because of uncertainty for a substance of the inhibitor, as far as we examined, it was an anionic substance from the results of experiments using ion exchangers, not precipitable by barium chloride,

and it was not sodium sulfite, sodium bisulfate, nitrite, ClO_3^- , Cl^- , SCN^- , Br^- and I^- . That the oxidation rate of Sigma's NADPH was very slower than that of Boehringer's NADPH may be elucidated in consideration of an unknown inhibitor in Sigma's NADPH.

To detection of the metabolic products, cell-free extracts of *P. vulgaris* was usually incubated with buffered glucose under anaerobic condition at 37°C for 4 hr. During such prolonged incubation, endogenous NADP might be destroyed completely by the action of NADP-pyrophosphatase in *P. vulgaris* (4). Accordingly, α -ketoglutarate and glycollate might be formed only a little or none, in spite of the supposition that large amounts of both the substances must be produced by only catalytic amount of NADP. Glycollate was detected eventhough it was only a little, while α -ketoglutarate could not. The latter, which must be produced at least corresponding with glycollate, was presumably converted to succinate *via* succinic semi-aldehyde (3).

Glyoxylate reductase has been reported to present in higher plants (9-15) and some bacteria (5, 16), but its physiological role seems to remain unexplained. In *P. vulgaris*, since endogenous oxidation of NADPH could not be observed, the enzyme might possibly play a role to protect excess reduction of intracellular NADP.

The enzyme is generally said to catalyze reduction of glyoxylate to glycollate. The reverse reaction, namely glycollate to glyoxylate, was not observed in *P. vulgaris*, nevertheless glycollate inhibited glyoxylate reduction. As seen in Fig. 6, however, the mode of the inhibition was non-competitive. Mechanism of glycollate inhibition has not been investigated concretely. Glyoxylate reductase in *P. vulgaris* from which NADP-pyrophosphatase was separated has been already practically used for determination of NADPH as a NADPH-oxidizing enzyme (4).

Summary

Glyoxylate reductase (EC 1.1.1.26) was confirmed to present in *Proteus vulgaris* by the following facts: The reaction product was proven to be glycollate by paper chromatography and spectrophotometry, and the amount of glycollate formed was stoichiometrically corresponding with that of glyoxylate consumed.

Optimal pH was found to be about 6.8. NADPH was required for the reaction, but it was not replaced by NADH. K_m values for NADPH and glyoxylate were 14.3 and 263 μM , respectively.

The reaction was inhibited intensely by an impurity in the sodium

hydrosulfite reagent, though it has not ascertained what is a substance of the inhibitor. That the chemically reduced NADPH (Sigma) was not rapidly oxidized by the enzyme might be due to suchlike impurity in the reagent.

The reaction was also inhibited by glycollate non-competitively.

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