On the Metabolism of D-Glucuronic Acid by *Pseudomonas ovalis*

Part I. Preliminary Research with Inhibitors*

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The enzyme systems concerning with the degradation of D-glucuronic acid by *Pseudomonas ovalis* 40F were investigated by the experiments using inhibitors for various enzyme systems. It was ascertained that the oxidation proceeded adaptively to the substrate and dehydrogenase systems containing DPN or Flavin as a coenzyme, oxidative decarboxylation and oxidative phosphorylation were presumed to participate in the oxidation of D-glucuronic acid by the phenomena of inhibition with fluoride, monoiodoacetate, arsenite, 2,4-dinitrophenol and azide to glucuronic acid oxidation. Moreover, the existence of succinodehydrogenase was also suggested by inhibition with malonate to succinic acid oxidation.

INTRODUCTION

Although it has been well known that D-glucuronic acid plays an important role in the antidotal action in the animal body and its antidotal mechanism has hitherto been studied, the metabolism of this sugar acid, itself, has yet been scarcely investigated. Above all, only a few reports have appeared in the past on oxidative metabolism of this acid by microorganisms.

Cohen¹⁾ reported that cells of *Escherichia coli* strain K12 adapted to glucuronic acid were capable of fermentation of uronic acids, but not homologous pentoses. According to Heald²⁾ the fermentation pattern of D-glucuronic acid by coli form bacteria was quite different from either that of homologous hexoses or pentoses. Furthermore, Heald³⁾ showed that cells grown on glucose formed adaptive enzyme systems for uronic acids, but these cells were not adapted to xylose in manometric experiments. In this latter paper it was noted that washed cell

suspensions of *Aerobacter aerogenes* grown on xylose did not ferment D-glucuronic acid. Whereas on the other hand, De Ley⁴⁾ found that *Aerobacter cloacae* oxidized D-glucuronic acid after short lag periods, indicating the formation of adaptive enzyme. In studies with cells or cell-free extracts of *Escherichia coli* strain K12 grown on D-glucuronic acid, Watanabe and Arai⁵⁾ demonstrated that D-glucuronic acid was first converted to D-galacturonic acid and then to 5-keto-L-galactonic acid. In contradiction to this work, Payne⁶⁾ found that prolonged culture of *Serratia marcescens* on either D-glucuronic or D-galacturonic acid yielded cells capable of oxidizing D-glucuronic acid rapidly and D-galacturonic acid slowly. From the behavior of the D-galacturonic acid grown cells, he suggested that D-galacturonic acid was isomerized to D-glucuronic acid before being metabolized by *S. marcescens*.

Sasty and Sarma⁷⁾ showed that *Aspergillus niger* was capable of utilizing D-glucuronic acid for the precursor of L-ascorbic acid.

The present work was undertaken with

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1) S.S. Cohen, *J. Biol. Chem.*, **177**, 607 (1949).

2) P.J. Heald, *Biochem. J.* (London), **50**, 503 (1952).

3) P.J. Heald, *Biochem. J.* (London), **52**, 378 (1952).

4) J. De Ley, *Enzymologia*, **16**, 14 (1953).

5) M. Watanabe and M. Arai, Lecture delivered at the annual meeting of Japanese Biochemical Society, Nov. 2 (1955).

6) W.J. Payne, *J. Bact.*, **72**, 834 (1956).

7) K.S. Sasty and P.S. Sarma, *Nature*, **179**, 44 (1957).

the view to obtain information referring to the mechanism of D-glucuronic acid oxidation by *Pseudomonas ovalis* as a part of series of studies on oxidative fermentation of bacteria.

EXPERIMENTAL

A strain of *Pseudomonas ovalis* 40F** isolated by Yokozawa⁸⁾ was selected as the test organism from about forty strains of *Acetobacter*, *Gluconobacter* and *Pseudomonas*. This strain is a facultative aerobe which oxidizes glucose by way of gluconic acid. It produces neither 2-keto-gluconic nor 5-keto-gluconic acid and has no ability to oxidize either of them. It is also remarkably different from another strain of *Pseudomonas* such as *Ps. aeruginosa* and *Ps. fluorescens liquefaciens* in this respect⁹⁾¹⁰⁾. Cells of *Acetobacter* and *Gluconobacter* tested were not induced to oxidize D-glucuronic acid, nor did extension of the period of incubation to 5 hours result in adaptation by them. Cultures were grown in shake culture on a reciprocal shaker at pH 5.8 in a medium containing 0.3% dried yeast extract powder (Oriental Yeast Manuf. Co.), 10% glucose and 2.5% calcium carbonate. The cells were harvested at a period of 18 hours, washed with 1.5% sodium chloride solution and distilled water. To prepare cells adapted to D-glucuronic acid, they were finally suspended at a concentration of 100 mg wet weight per ml in M/20 phosphate buffer (pH 6.0) containing 0.7% sodium D-glucuronate and were incubated at 30°C for 5 hours. Cells adapted were rewashed as described above and taken up in distilled water to give a suspension of about 25 mg dry weight per ml (except those employed in the experiments shown in Fig. 1). Oxygen uptake and CO₂ evolution were measured by the Warburg apparatus at 30°C. All the values of endogenous respiration were subtracted. Each manometer cup was charged with one ml of M/20 phosphate buffer (pH 6.0, except in case to test the effect of pH), 0.5 ml of substrate (5 μ M), 0.5 ml of bacterial suspension in the side arm and 0.2 ml of 20% potassium hydroxide in the center

well. Moreover, a portion of 0.5 ml of aqueous solution of the inhibitor (whose concentration was in the figures respectively) or an equal volume of water was added to the cup in the experiments in order to examine the effects of inhibitors. Inhibitors used in this study were sodium fluoride, monoiodoacetic acid, sodium arsenite, 2,4-dinitrophenol, sodium azide and malonic acid. Monoiodoacetic and malonic acids were neutralized with sodium hydroxide before being employed. They were all extra-pure products obtained commercially.

RESULTS AND DISCUSSION

Washed cells of *Ps. ovalis* 40F grown on glucose exhibited adaptation to D-glucuronic acid after incubation for 2 hours (Fig. 1, curve A) like *E. coli*¹⁾⁵⁾, *coli form bacteria*³⁾,

FIG. 1. Adaptive Curves of D-Glucuronic Acid Oxidation.

*Aero. cloacae*⁴⁾ and *S. marcescens*⁶⁾ investigated hitherto did. Reaction mixtures used in the experiment by which curve A in Fig. 1 was obtained were centrifuged to collect the cells. The cells were used for measuring the oxygen uptake and resulted curve B in Fig. 1. In this latter experiment D-glucuronic acid was oxidized without a lag period and linearly to incubation period. The rate of oxidation of D-glucuronic acid by adapted cells increased up to several times to that of the cells

** This strain was formerly designated as *Ps. fluorescens* 40F by Yokozawa, however, it has been corrected as *Ps. ovalis* according to the exact identification by Shoda in our laboratory.

8) K. Yokozawa, *J. Agr. Chem. Soc. Japan*, **25**, 556 (1951).

9) J.J.R. Campbell and F.C. Norris, *Canad. J. Research*, C28, 203 (1950), F.N. Stokes and J.J.R. Campbell, *Arch. Biochem. Biophys.*, **30**, 121 (1950).

10) T. Asai and H. Murooka, *J. Agr. Chem. Soc. Japan*, **30**, 1 (1956).

before adaptation occurred.

The effect of pH to the oxidation of D-glucuronic acid is shown in Fig. 2. Although

FIG. 2. Optimum pH in the Oxidation of D-Glucuronic Acid.

optimal pH was found to be approximately 7, little difference on enzyme activities was observed between pH 7 and pH 6 or 8. Thus, pH 6 was conventionally selected in the experiments hereafter.

The QO_2 value for D-glucuronic acid was about 25–30 and RQ value was 1 at these pH conditions.

The metabolism of D-glucuronic acid in microorganisms has scarcely been investigated, and especially, its terminal pathway has been

FIG. 3. Inhibition to D-Glucuronic Acid Oxidation with NaF.

completely unclarified. Therefore, the inhibition phenomena to various enzyme systems with inhibitors were investigated to presuppose it.

Sodium fluoride at a concentration of 5×10^{-2} M completely inhibited the oxidation of D-glucuronic acid (Fig. 3). Oxygen uptake to D-glucuronic acid by intact cells was perfectly suppressed with sodium moniodoacetate at a minimum concentration of 5×10^{-3} M (Fig. 4). Therefore, the evidence

FIG. 4. Inhibition to D-Glucuronic Acid Oxidation with ICH₂COOH.

was supported that dehydrogenase systems contained DPN or Flavin as coenzyme might participate in the oxidation of D-glucuronic acid.

Sodium arsenite showed a remarkable inhibition at a concentration of 10^{-3} M (Fig. 5). Since arsenite inhibits oxidative decarboxylation, the existence of oxidative decarboxylation was readily assumed from this experiment.

At 10^{-3} M 2,4-dinitrophenol and at 10^{-2} M sodium azide concentrations, perfect inhibitions to D-glucuronic acid degradation were observed (Figs. 6 & 7). Both inhibitors are reagents for suppression to the oxidative

Fig. 5. Inhibition to D-Glucuronic Acid Oxidation with Arsenite.

Fig. 6. Inhibition to D-Glucuronic Acid Oxidation with 2,4-DNP.

phosphorylation in the enzyme reaction sequence. Watanabe and Arai¹¹⁾ presumed that the phosphorylation in D-glucuronic acid metabolism by *E. coli* strain K12 would be occurred at the 6-position of carbon of 5-keto-L-galactonic acid. Although it was not clarified where the phosphorylation did

11) M. Watanabe and M. Arai, Lecture delivered at the Symposium of Enzyme Chemistry, July 24 (1956).

Fig. 7. Inhibition to D-Glucuronic Acid Oxidation with NaN_3 .

occur, it would be said that the phosphorylation took place somewhere in the metabolic pathway of D-glucuronic acid oxidation by *Ps. ovalis*.

Sodium malonate at a minimum concentration of 10^{-2} M suppressed the oxidation of succinic acid by the resting cells of *Ps. ovalis* (Fig. 8). The incomplete inhibition might be caused by the permeability of malonate

Fig. 8. Inhibition to Succinate Oxidation with Malonate.

to succinodehydrogenase in washed cells.

Since D-glucuronic acid did not produce any oxidation product by *Ps. ovalis* 40F, it might be completely oxidized. It might be caused by oxidative assimilation that the values of oxygen uptake by intact cells did not coincide to the values calculated theoretically according to the formula of complete oxidation. From this and the results obtained above, it was supposed that D-glucuronic acid

was finally metabolized to CO_2 and H_2O through Krebs' TCA cycle in a manner like that observed in the glucose oxidation by another *Pseudomonas* such for instance as *Ps. aeruginosa*, *Ps. fluorescens*. Further details concerning with this problem would be investigated in the following later paper.

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