**138. Studies on Oxidative Fermentation. XV. On the
Chemical Pathway Involved in the Formation of
 γ -Pyrone Derivatives from Glucose by
*Gluconoacetobacter liquefaciens***

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(Comm. by T. YABUTA, M.J.A., Oct. 12, 1956)

In the previous papers,¹⁾⁻³⁾ the authors reported on the formation of three γ -pyrone derivatives from glucose by *Gluconoacetobacter liquefaciens* (syn. with *Acetobacter melanogenum*). One of them was identified as comenic acid, while other two substances were found to be new γ -pyrone derivatives which were named rubiginol and rubiginic acid, and their chemical compositions were decided to be 3,5-dihydroxy-1,4-pyrone and 3,5-dihydroxy-1,4-pyrone-2-carboxylic acid respectively. As the formation of γ -pyrone derivatives by microorganisms is very interesting from the viewpoint of the microbial sugar metabolism, detailed studies concerning with this problem have been conducted, and the most presumable pathway resulting in the formation of these γ -pyrone derivatives is presented.

Experimental

Gluconoacetobacter liquefaciens, which had been isolated by T. Asai,⁴⁾ was used as the strain throughout the experiments.

I. Formation of γ -pyrone derivatives from various carbon sources. 10 ml portions of the medium composed of 0.8 % yeast extract water and 5 % carbon sources were dispensed in inverse T-shape shaking tubes, to which sterilized CaCO_3 equivalent to one fourth of carbon sources was added just before inoculation. Organic acids were used as sodium salts without addition of CaCO_3 . The incubation was carried out on a reciprocating shaker for 10 days at 30°C. The above-mentioned three γ -pyrone derivatives were confirmed to be formed from only glucose, gluconate, and 2-ketogluconate, by paper chromatography, among thirty kinds of carbohydrates and alcohols, and fifteen kinds of organic acids examined.

II. Oxidation of glucose, gluconate, and 2-ketogluconate by intact cell suspension and cell-free extracts. Manometric experiments were carried out with the Warburg manometer. As shown in Fig. 1, it is postulated that all substrates were oxidized rapidly with the evolution of CO_2 , while, in the presence of $2 \times 10^{-3}\text{M}$ 2,4-dinitrophenol (DNP), CO_2 evolution was not observed, and 1.5, 1.0, and 0.5 moles of

Fig. 1. Oxidation of glucose, gluconate, and 2-ketogluconate by intact cell suspension of *Gluconoacetobacter liq.*

Each vessel contained 0.5 ml of the cell suspension, 1 ml of phosphate buffer solution (M/15, pH 6.0) and 5 μ M of the substrate. Total volume, 25 ml. Temperature, 30°C

Oxygen uptake: I...glucose, II...gluconate, III...2-ketogluconate

Carbon dioxide evolution: IV...glucose, V...gluconate, VI...2-ketogluconate

Fig. 2. Effect of 2,4-dinitrophenol

I...Glucose (oxygen uptake)

II...Glucose (carbon dioxide evolution)

III...Glucose + DNP (oxygen uptake)

IV...Gluconate + DNP (oxygen uptake)

V...2-Ketogluconate + DNP (oxygen uptake)

oxygen were consumed per mole of glucose, gluconate, and 2-ketogluconate respectively (Fig. 2). When cell-free extracts were used instead of intact cells, the evolution of CO_2 did not occur and the oxygen uptake of each substrate was essentially the same as the case observed in the experiment of intact cell suspension in the presence of DNP. This phenomenon demonstrates strongly that the oxidation of glucose should proceed via gluconate and 2-ketogluconate, and moreover the evolution of CO_2 might not occur during these steps of oxidation. NaF ($5 \times 10^{-2}\text{M}$) did not influence either on the rate or on the total amounts of oxygen uptake with glucose. The oxidation of glucose, gluconate or 2-ketogluconate was not depressed to any extent in phthalate buffer and also glucose-1-phosphate, glucose-6-phosphate, or 6-phosphogluconate was scarcely oxidized. These results suggest that glucose may not be phosphorylated prior to further dissimilation.

III. Formation and identification of 2,5-diketogluconate from 2-ketogluconate. *Gluconoacetobacter liquefaciens* was grown in shaking flask at 30°C for 40 hours. The cells were centrifuged, washed with 0.85 % NaCl solution and distilled water, then they were dried in

desiccator for one night. 500 mg of Ca-2-ketogluconate was dissolved in 250 ml of distilled water and was dispensed in five shaking flasks of 500 ml capacity. 100 mg of dried cells was added to each flask and incubated at 30°C for 30 minutes on a reciprocating shaker. Clear supernatant was lyophilized immediately and then it was dissolved in a small quantity of water and precipitated by the addition of ethanol for three times. About 400 mg of pale-yellow Ca salt was obtained. The absorption spectra of purified Ca salt are shown in Fig. 3. $\lambda_{\max}$ at pH 5.7 and at pH 1.0 were observed to be 305 m μ and 280 m μ respectively. Raising the pH up to 9.0, it showed $\lambda_{\max}$ of 305 m μ . These figures and shapes of absorption spectra quite agreed with those of Ca-2, 5-diketogluconate which had been isolated by Katznelson et al.,⁵⁾ and the R_F value of the spot in descending paper chromatography, using the solvent system of water saturated isobutyric acid and sprayed with aniline hydrogen oxalate, showed 0.54, while those of 2-ketogluconate and 5-ketogluconate were 0.77 and 0.87 respectively. From these data this substance was identified as 2, 5-diketogluconic acid.

IV. Decomposition of 2,5-diketogluconate by cell suspensions. 100 mg of the substrate was dissolved in 100 ml M/15 phosphate buffer (pH 6.0) and was taken in 500ml shaking flask, to which 500 mg of intact cells was added and incubated at 30°C for 5 hours. In the centrifuged supernatant, the existence of three γ -pyrone derivatives corresponding to comenic acid, rubiginic acid, and rubiginol besides glycolic and tartronic acids was detected by paper chromatography.

V. Formation of rubiginol from rubiginic acid. 10 ml portions of the medium composed of 0.8% yeast extract water and 1% mannose was dispensed in two inverse T-shape shaking tubes, one of which contained 100 mg of rubiginic acid. 25mg CaCO₃ sterilized separately was added before inoculation. The cultures were grown for 10 days at 30°C on a reciprocating shaker. From the broth which contained rubiginic acid, rubiginol was detected by paper chromatography, contrary to the blank experiment in which the formation of any substance of positive ferric chloride reaction was denied.

Fig. 3. Ultraviolet absorption spectra of the oxidation product from Ca-2-ketogluconate by dried cell preparation of *Gluconoacetobacter liq.*

Discussion and Results

We could not find the formation of any γ -pyrone derivatives from C_2 or C_3 compound, and comenic acid, rubiginic acid, and rubiginol were found to be formed only from glucose, gluconate, 2-ketogluconate, and 2,5-diketogluconate.

Therefore, the three γ -pyrone derivatives were assumed to be formed via direct oxidative pathway as shown in the following scheme.

Fig. 4

As in the case of the oxidation of glucose by notation,⁶⁾ where gluconolactone is the primary product and with the similar oxidation of glucose-6-phosphate to 6-phosphogluconolactone, it is tempting to postulate that the first stage may be the removal of two hydrogen atoms from carbon atom 1 of glucopyranose. Such oxidation, i.e., $CHOH \rightarrow CO$, occurs frequently in fungal and bacterial metabolisms as shown by the common occurrence of ketogluconic acids, although usually only the 2- or 5-keto compound is formed. Gluconolactone converts to gluconic acid, and the latter is oxidized to 2-ketogluconic and 2,5-diketogluconic acid. The formation of 5-ketogluconate was also observed in this experiment. 2-Ketogluconate is further oxidized to 2,5-diketogluconate. The formation of enol form of 2,5-diketogluconate was suggested by the reduction of 2,6-dichlorophenol-indophenol. Katznelson et al.,⁵⁾ also described that the fact of the absorption maximum shifts towards lower wave-length upon acidification might be due to the association with enediol compounds such as ascorbic and dihydroxymaleic acids.

Concerning the formation of glycolic acid, it can be easily supposed

to be derived from 2,5-diketogluconate by direct splitting at the point of CO group.

As reported before,⁷⁾ the unknown reducing organic acid was also found to be formed from glucose with a fairly high yield by this bacteria, and it is supposed to be an intermediate product of main pathway of glucose oxidation, although the product is not identified yet.

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