Pathways of 5-ketogluconate catabolism in bacteria*

5-Ketogluconate is a biochemical curiosity. In nature, it is formed only by *Acetobacter* species of the *suboxydans* and *mesoxydans* group, often in copious amounts, during the oxidation of glucose and gluconate. Little is known concerning its further metabolism[1,2,3].

Both 2- and 5-ketogluconic acids may also be considered to be uronic acids, derived from the corresponding ketoses D-fructose and L-sorbose, by oxidation of one $-CH_2OH$ group. It would not be surprising to find that the first step in the metabolism of all uronates follows the same principle. Indeed, from previous experiments on the metabolism of glucuronate, galacturonate and 2-ketogluconate (D-fructo-1-uronate) by bacteria, it appears that this primary reaction consists of a reduction of the $-CHO$ or $>C=O$ group. Glucuronate and galacturonate are converted into the corresponding L-hexonates by a TPNH- or DPNH-linked uronate reductase from *Erwinia* and *Aerobacter*[4]; 2-keto-6-phosphogluconate (fructo-1-uronate-6-phosphate) is converted into gluconate-6-phosphate by a TPNH-linked reductase from *Aerobacter cloacae*[5]. 2-Ketogluconate is reduced by a similar enzyme[5]. We therefore postulated that the catabolism of 5-ketogluconate (L-sorbo-6-uronate) could likewise proceed after a preliminary coenzyme-linked reduction.

Enrichment cultures were set up at 30° in a medium containing 0.1 % yeast extract; 0.5 % KH_2PO_4; 0.15 % $(NH_4)_2SO_4$; 0.5 % NaCl; 0.025 % $FeSO_4$; 0.025 % $MgSO_4 \cdot 7H_2O$; 0.02 *M* sodium 5-ketogluconate; pH 7.2, inoculated with soil, mud, river and ditch water. By the usual procedures, eight strains of bacteria were isolated, which all grew very well on 5-ketogluconate as sole carbon source and consumed it completely. Three strains were identified as *Klebsiella* species (KAUFMAN's nomenclature[6]), three as *Escherichia coli*, and the two other strains have temporarily been classified as *Alcaligenes*. Each of these strains was grown aerobically either on yeast extract and peptone or on a synthetic medium containing either glucose, gluconate or 5-ketogluconate. The resting cells were used in the Warburg apparatus for the study of the respiration curve of several substrates. In every case glucose was oxidized with a set of constitutive enzymes. 2-Ketogluconate and D-xylulose were oxidized after an induction period. 5-Ketogluconate was oxidized at once only with cells grown on this substance; cells grown on other substrates oxidized it only after an induction period. However, gluconate was oxidized at once with both gluconate- and 5-ketogluconate-grown cells, and after an induction period with cells grown under other conditions. These results show that gluconate and 5-ketogluconate are not intermediates in the glucose oxidation by these bacteria. The sequential induction of 5-ketogluconate-grown cells to gluconate indicates that 5-ketogluconate may indeed be metabolized after a previous reduction, as the main physiological pathway.

Cell-free extracts from 5-ketogluconate-grown cells were prepared either by grinding with alumina or by disruption in the Hughes press, extracting with 0.01 *M* NH_4Cl–NH_4OH buffer, pH 10, followed by high-speed centrifugation (100,000 *g* for 2 h at 4°) and dialysis. All the strains contained an enzyme for the reduction of 5-ketogluconate by DPNH. This enzyme was very active in *Klebsiella* and *Escherichia*, but less so in *Alcaligenes*. *Klebsiella* 5K3B and *Escherichia* 5K2C were selected for further study of the following reactions:

$$\text{5-ketogluconate} + \text{DPNH} + H^+ \rightleftharpoons \text{gluconate} + \text{DPN}^+ \quad (1)$$

$$\text{5-ketogluconate} + \text{TPNH} + H^+ \rightleftharpoons \text{gluconate} + \text{TPN}^+ \quad (2)$$

Reaction (1) is about twice as fast as (2). The enzyme is active between pH 4 and 11. The reaction is reversible: at pH 6–7 the reaction is heavily shifted to the right, while at pH 10–11 the reverse reaction occurs (Fig. 1). When 4 μmoles 5-ketogluconate are incubated with an equimolar amount of either DPNH or TPNH and 0.1 ml enzyme in 3 ml 0.02 *M* tris(hydroxymethyl)aminomethane buffer, pH 7.3, gluconate could be identified in both cases by the conversion into the lactone, by the reaction with the hydroxylamine reagent[7] and by paper chromatography in the upper phase of a mixture of butanol–ethanol–water (4:1:5). Idonate, the other possible end product after 5-ketogluconate reduction, was not detectable. To demonstrate the end product in the reverse reaction, a system containing 50 μmoles sodium gluconate, 2 μmoles DPN or TPN, 0.1 ml enzyme in 3 ml 0.02 *M* glycocoll buffer, pH 10, was incubated for 1–2 h at room temperature, decationized with Amberlite IR-120 H^+, dried *in vacuo* and chromatographed on paper for 48 h in the upper phase of a mixture of *n*-propanol, methylbenzoate, HCOOH and water (7:3:2:5). After spraying with *o*-phenylenediamine[8], a spot was revealed which had the typical blue color and the same position as an authentic sample of 5-ketogluconate. The stoicheiometry of the reactions (1) and (2) in both directions could also be shown spectrophotometrically and by estimation of 5-ketogluconate with the method of SCHRAMM[9]. This enzyme preparation did not reduce 2-ketogluconate, fructose or L-sorbose. We propose the name "5-ketogluconoreductase".

* This work was supported in part by a grant from the Nationaal Fonds voor Wetenschappelijk Onderzoek.

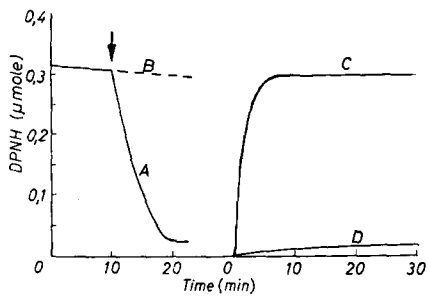

Fig. 1. Reversibility of the 5-ketogluconoreductase reaction in *Klebsiella* 5K3B extract as measured in the Beckman spectrophotometer model DU. Left part: reaction of 5-ketogluconate with DPNH. The system contained in 3 ml 0.02 *M* tris (hydroxylmethyl)aminomethane–HCl buffer, pH 7.2 : 0.3 μmole DPNH; 0.025 ml enzyme; 2 μmoles 5-ketogluconate (curve A) or water, D-fructose, L-sorbose or 2-ketogluconate (curve B) is added at the arrow. Right part: reaction of gluconate with DPN. The system contains in 3 ml 0.02 *M* glycocoll buffer, pH 10; 50 μmoles sodium gluconate; 0.3 μmole DPN and 0.1 ml enzyme (curve C). Curve D: without gluconate.

However, there is still another pathway for 5-ketogluconate metabolism. By the manometric method of COLOWICK AND KALCKAR[10], a 5-ketogluconokinase was detected. 5-Ketogluconate did not disappear in the absence of ATP. The kinase was present in greatest activity in the *Klebsiella*'s. It could hardly be detected in the other strains. Concomittant analysis of 5-ketogluconate disappearance and CO_2 formation during the phosphorylation showed that about 2 moles of CO_2 are liberated per mole substrate metabolized. The reaction thus proceeds beyond the primary phosphorylation, which we assume to be the formation of 5-keto-6-phosphogluconate. A calculation of the relative importance of either pathway, based on the enzyme activities in the extracts, showed that 5-ketogluconate is metabolized about 300 times as fast by the reductive pathway as by the phosphorylative. That the former pathway is physiologically the more important in our bacteria follows also from the above mentioned experiments on the sequential induction pattern of resting cells. Work on the mechanism of the phosphorolytic pathway is in progress.

Biochemical Laboratory, Veterinary School, State University, Ghent (Belgium) J. DE LEY*

[1] J. A. FEWSTER, *Biochem. J.*, 63 (1956) 26 P.
[2] J. A. FEWSTER, *Biochem. J.*, 65 (1957) 14P.
[3] J. A. FEWSTER, *Biochem. J.*, 66 (1957) 9 P.
[4] M. P. STARR, J. DE LEY AND W. KILGORE, *Science*, 125 (1957) 929.
[5] J. DE LEY AND S. VERHOFSTEDE, *Enzymologia*, 18 (1957) 47.
[6] F. KAUFMAN, *Enterobacteriaceae*, E. Munksgaard, Copenhagen (1954).
[7] S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 249.
[8] M. C. LANNING AND S. S. COHEN, *J. Biol. Chem.*, 189 (1951) 109.
[9] M. SCHRAMM, *Anal. Chem.*, 28 (1956) 963.
[10] S. P. COLOWICK AND H. M. KALCKAR, *J. Biol. Chem.*, 148 (1943) 117.

Received November 2nd, 1957

* Associé of the Nationaal Fonds voor Wetenschappelijk Onderzoek.

The path of electrons in the respiratory chain of enzymes

Kinetic studies of the respiratory enzymes of whole wheat roots and baker's yeast[1,2,3] have shown that the main enzymes, viz. cytochromes a_3, *c* and *b*, flavoprotein (FP) and diphosphopyridine nucleotide (DPN), are reduced in a characteristic time order, if the cells or tissues are exposed to anaerobiosis. One experiment with yeast yielded the following figures of the start times following a shift from air to N_2:

a_3	*c*	*b*	FP	DPN
0.5	1.0	8.2	7.4	24.7 sec

The reoxidation (shift from N_2 to air) runs considerably faster. It could be shown, however, that the time order is now reversed, *i.e.* cytochrome *b* is more rapidly oxidized than cytochrome *c*, etc. As shown from continuous records of the spectral bands at increasing reduction after anaerobiosis, the group cytochromes a_3–*c* is nearly reduced before the group cytochrome *b*–FP starts reduction, and DPN starts reduction about simultaneously with the completed reduction of cytochrome *b*–FP.