THE MECHANISM AND LOCALIZATION OF HEXONATE METABOLISM IN *ACETOBACTER SUBOXYDANS* AND *ACETOBACTER MELANOGENUM*

J. DE LEY* AND A. J. STOUTHAMER

Biochemical Laboratory, Veterinary School, State University, Ghent (Belgium) and Laboratory for Hygiene, State University, Utrecht (The Netherlands)

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SUMMARY

In our strain of *Acetobacter suboxydans*, three enzymes are present for the oxidation of gluconate:

(1) a soluble TPN-specific dehydrogenase, yielding 2-ketogluconate (2-ketogluconoreductase);

(2) a soluble TPN-specific dehydrogenase, yielding 5-ketogluconate (5-ketogluconoreductase);

(3) a particulate, possibly cytochrome-linked gluconate oxidase, yielding 2-ketogluconate.

The specificity of these enzymes was studied. Galactonate is oxidized with a TPN-linked dehydrogenase to 2-ketogalactonate. Soluble TPN-linked dehydrogenases for glucose, mannose, galactose, xylose and L-arabinose are present. A 5-ketogluconokinase was not detectable. Both 2- and 5-ketogluconates are metabolized by a reductase into gluconate, followed by a gluconokinase and a 6-phosphogluconodehydrogenase.

A strain of *Acetobacter melanogenum* was also investigated because in a period of one year it had lost the power to make a brown pigment and now produced considerable amounts of Ca-5-ketogluconate. The new behaviour of this strain was due to the fact that the 2-ketogluconoreductase was absent and the gluconate oxidase content was low. 5-Ketogluconate is not metabolized by this strain, owing to the absence of a gluconokinase.

INTRODUCTION

Growing cultures of acetic acid bacteria of the suboxydans and mesoxydans group produce considerable amounts of both 2- and 5-ketogluconate during the oxidation of glucose and gluconate[1-6]. *Acetobacter suboxydans*[1] is undoubtedly the prototype, producing copious amounts of calcium 5-ketogluconate** crystallizing out in the

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** The following abbreviations will be used: 5-ketogluconate (5K); 2-ketogluconate (2K); diphosphopyridine nucleotide (DPN); triphosphopyridine nucleotide (TPN); adenosine triphosphate (ATP); glucose-6-phosphate (G6P); gluconate-6-phosphate (6PG).

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medium. However, little is known about the enzymic mechanism of the formation and of the further metabolism of both keto-acids in these species. *A. melanogenum* is unique in that it oxidizes 2K with the formation of 2,5-diketogluconate[7], which is then degraded with the formation of α-ketoglutarate via pentoses[8]. There are a few indications in the literature that in some *Acetobacters* both ketohexonates may be intermediates in separate pathways of glucose breakdown, different from those previously described, such as the glycolysis cycle, the hexose monophosphate oxidative cycle (shunt) or the Entner-Doudoroff mechanism. For example, JACKSON *et al.*[9] mentioned briefly that various *Acetobacters* may produce *d*-tartaric and oxalic acids from 5-ketogluconate and that the oxidation of gluconate results in the production, first of 2-ketogluconate and then of pyruvate and CO_2. FEWSTER[10] reported that resting cells of *Acetobacter suboxydans* ATCC 621 oxidize 5-ketogluconate with an O_2 consumption of 3.5 moles/mole substrate and 2-ketogluconate with the uptake of 0.5 mole O_2. FEWSTER[11] mentioned that D-xylulose and both 2- and 5-ketogluconate are phosphorylated. It was suggested that 5-ketogluconate was decarboxylated to D-xylulose. Since D-xylulose-5-phosphate is an intermediate in the hexose-monophosphate oxidative cycle, the nearly complete oxidation of 5-ketogluconate could be explained. However, experimental evidence for a conversion of 5K into D-xylulose was not given. From his experiments FEWSTER[10] concluded "that gluconate and the two oxo acids are intermediates of a main pathway for the extensive aerobic metabolism in *Acetobacter suboxydans*".

In a paper, published after the present manuscript was ready, FEWSTER[12] reports that his strain also oxidizes 2K with the uptake of 0.5 mole O_2 per mole substrate, the production of 0.5 mole CO_2 and the apparent formation of arabonate.

For the present investigations, a strain of *Acetobacter suboxydans* was selected which was able to oxidize the ketohexonates, accumulating in the medium in the initial growth period, with the formation of CO_2. It was therefore expected that the enzyme systems for the new types of catabolism might be present. It was found, however, that the primary step in the metabolism of both ketogluconates consists in a reduction to gluconate, which is then phosphorylated by the gluconokinase. The gluconate-6-phosphate formed is then metabolized by the HMP oxidative cycle, which was shown to be present in our strain. It had previously already been demonstrated in another strain of *Acetobacter suboxydans* by HAUGE, KING AND CHELDELIN[13].

Thus the metabolism of 5-ketogluconate in this species follows the same pathway as in *Klebsiella* and *Escherichia*[14,15], and 2-ketogluconate is metabolized in the same way as in *Corynebacterium helvolum*[16]. Further it will be shown that gluconate is oxidized by 3 enzymes:

(1) 2 soluble TPN-linked dehydrogenases, one yielding 5-ketogluconate and one yielding 2-ketogluconate;

(2) a particle-linked, coenzyme-independent oxidase yielding 2-ketogluconate.

EXPERIMENTAL

1. The strain of *Acetobacter suboxydans* was isolated from beer and identified according to FRATEUR[5]. It differed only in one aspect from the strain described by this author as *Acetobacter suboxydans* (KLUYVER AND DE LEEUW): when growing on yeast–glucose–$CaCO_3$ slants, the bacteria formed first acid and crystals of Ca-5-keto-

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gluconate; later $CaCO_3$ was formed again, giving a strong irisation. This indicates that the acids are completely oxidized.

2. Mass cultures were grown on 2 different media. Roux flasks were used, containing a solid medium with 10 % glucose, 1 % yeast extract, 2½ % agar and 3 % $CaCO_3$ (in suspension). They were inoculated and incubated at 30° for either 48 to 72 h. The cells were then washed from the agar surface with $M/100$ phosphate buffer pH 6.0. We also used a liquid medium, containing 2 % Ca-gluconate, 0.2 % $NH_4H_2PO_4$, 0.1 % K_2HPO_4, 0.025 % $MgSO_4$ and 0.5 % yeast extract, dispensed in Erlenmeyer flasks in shallow layers. After inoculation, the flasks were incubated at 30° in a shaking-machine for 48 or 72 h. The culture was then freed from the precipitated Ca-5-ketogluconate by decantation and filtration. The cells from both media were harvested by centrifugation and were washed twice with $M/100$ phosphate buffer pH 6.0.

3. Cell-free extracts were prepared by treating the cell suspension (9–12 g in 50 ml $M/100$ phosphate buffer pH 6.4), in a 10 KC, 250 W Raytheon Sonic Oscillator for 15 min.

The suspension was then centrifuged for 1 h at 12,000 rev./min in an M.S.E. centrifuge at 4°. The precipitate was discarded and the supernatant was called "crude cell-free extract". It was centrifuged for 2 h at 105,000 *g* and 0° in a Spinco refrigerated preparative ultracentrifuge. In this way it was separated into a clear yellow solution, containing the soluble enzymes, called "particle-free supernatant" and a reddish precipitate, containing the bacterial particles. Sometimes the supernatant was dialyzed at 4° against $M/100$ phosphate buffer pH 6.0 for 18 h. 2–3 ml of the sedimented particles were suspended in 5 ml of $M/100$ phosphate buffer pH 6.0 with the aid of a Potter-Elvehjem homogenizer.

4. For manometric experiments the conventional Warburg respirometer was used at 30°.

The oxidations by resting cells were studied with 2 ml cell suspension in $M/15$ phosphate buffer pH 6.0 (about 100–150 mg living cells): 5 μmoles of the substrate in 0.2 ml were tipped in from the side-arm, except for Na-5-ketogluconate, of which 8.1 μmoles were used. The center wells contained 0.15 ml KOH solution. The gas phase was air.

Kinases were detected by the method of COLOWICK AND KALCKAR[17]. The main compartment of the Warburg vessel contained: 0.6 ml particle-free supernatant (from about 100 mg living cells), dialyzed or undialyzed as indicated; 10 μmoles substrate; 40 μmoles $NaHCO_3$, 16 μmoles $MgCl_2$; 20 μmoles NaF. 17 μmoles of Na-ATP in a volume of 0.2 ml 0.02 M $NaHCO_3$ were added from the side-arm. The total volume was 2 ml. The gas phase was 95 % N_2 + 5 % CO_2, final pH 7.4.

The oxidation of the different substrates by the particle-free supernatant was tested in the following system: 0.3 ml enzyme preparation; 36 μmoles Tris buffer pH 7.4; 36 μmoles $MgCl_2$; 0.65 mg N-methylphenazinium methylsulfate; 0.3 μmole DPN or TPN; 20 μmoles substrate in a total volume of 1.9 ml. The center well contained 0.1 ml 20 % KOH solution. The gas phase was air.

Oxidations with the particles were carried out in $M/30$ phosphate buffer pH 6.0, corresponding to about 0.1 ml packed particles, with 20 μmoles substrate.

5. Spectrophotometric determinations were carried out with the Beckman apparatus, model DU, at about 20°. Dehydrogenase activity was estimated by reading

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the optical density at 340 mμ. The system contained: 60 μmoles Tris buffer pH 7.4; 60 μmoles $MgCl_2$; 0.3 μmoles TPN; 2 μmoles substrate (ethanol, Na-G6P or Na-6PG) and 0.3 ml particle-free supernatant in a total volume of 2.9 ml.

The enzymic reduction of ketogluconates by TPNH was studied in the following way: TPNH was generated by one of the dehydrogenases just mentioned, 10 μmoles of the ketogluconates in 0.1 ml were added to the same system and the decrease in the O.D. followed at 340 mμ. The oxidation of hexonates and pentonates with either TPN or DPN was demonstrated in a system containing: 60 μmoles glycine buffer pH 10; 50 μmoles substrate; 0.3 μmole coenzyme and 0.3 ml particle-free supernatant in a total volume of 3.0 ml.

6. To detect the endproducts of the phosphorylation of 5-ketogluconate by ATP the following reaction mixture was incubated at 30°; 67 μmoles phosphate buffer pH 7.4; 16 μmoles $MgCl_2$; 20 μmoles NaF; 17 μmoles ATP; 13 μmoles 5-ketogluconate; 0.3 μmole TPN and 0.4 ml dialyzed particle-free supernatant in a total volume of 2 ml. At regular intervals a 0.1 ml sample was withdrawn, with which the cysteine–carbazol reaction[18] and the CyR1 reaction[19] was carried out. 5-Ketogluconate gave a purplish colour in the cysteine–carbazol reaction. However, the optical densities at 540 and 560 mμ were low. In the CyR1 reaction 5-ketogluconate yields a green colour with a broad absorption maximum at 540 mμ.

7. Ketogluconates were detected by descending paper chromatography for 48 h in the upper phase of a mixture of *n*-propanol, methylbenzoate, formic acid and water (7:3:2:5). The *o*-phenylenediamine spray[20] was used to reveal the position of the ketogluconic acids. After heating for 3 min at 95–100°, 2-ketogluconate gives a green spot with yellow-green fluorescence and 5-ketogluconate gives a blue spot without fluorescence. 2-Ketogluconate was determined by the reaction with *o*-phenylenediamine–HCl, according to the method of LANNING AND COHEN[20] and 5-ketogluconate by the reaction with 1-methyl, 1-phenylhydrazine sulfate according to the method of SCHRAMM[21].

RESULTS

1. *Experiments with resting cells*

Fig. 1 represents the result of a typical experiment with resting cells. Glucose, gluconate, 2- and 5-ketogluconate are oxidized with an O_2 consumption of 60–70% of the amount needed for complete oxidation. Since our strain oxidizes 2-ketogluconate with an O_2 consumption of 3.4 moles/mole substrate, it is different from *Acetobacter suboxydans* ATCC 621, used by FEWSTER, which consumed only 0.5 mole[10,12]. Ethanol is oxidized readily with an O_2 consumption of 1 mole/mole substrate, as expected for an *Acetobacter suboxydans*.

Table I summarizes the results of the oxidations, carried out by resting cells. Galactose was slowly oxidized; the O_2 consumption was always greater than 0.5 mole/mole substrate. Galactonate was also slowly oxidized. With galactose and galactonate the endpoint of the oxidation was never reached.

2. *Enzymes in the particle-free supernatant*

(*a*) *Dehydrogenases.* The particle-free preparation contains several coenzyme-linked dehydrogenases for the oxidation of some aldoses, hexonates, ethanol, G6P and 6PG.

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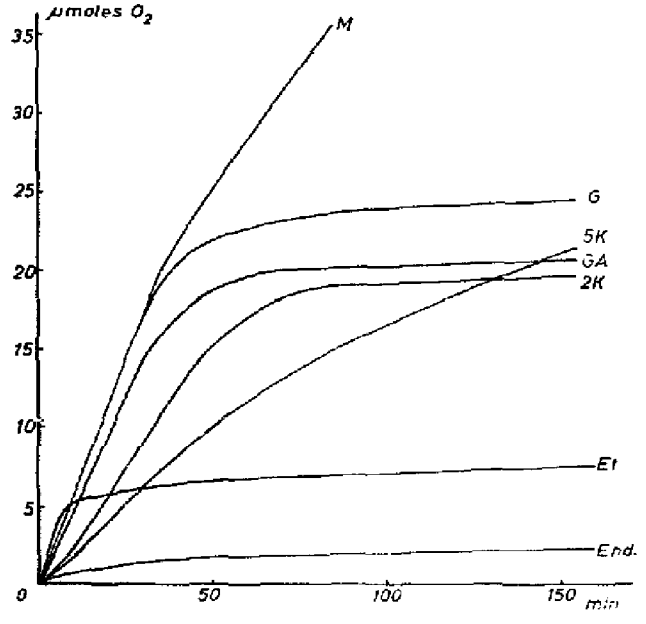
In our strain a glucose dehydrogenase is present, which is absolutely TPN-specific. This enzyme seems to be different from the DPN-specific one described by KING AND CHELDELIN[22] in another strain of *Acetobacter suboxydans*.

TABLE I

O_2 UPTAKE BY RESTING CELLS OF *Acetobacter suboxydans* WITH SEVERAL SUBSTRATES

Substrate	*Mole O_2/mole substrate*	
Glucose	4.4	3.9
Gluconate	3.7	3.4
5-Ketogluconate	2.8	3.2
2-Ketogluconate	3.5	3.3
Fructose	4.0	4.4
Galactose		$>$ 0.53
Galactonate		$<$ 0.2
Maltose		$>$ 6.7
D-Xylose		0.5
L-Arabinose		0.4
D-Mannose		0.5
Ethanol	1.0	0.9
Pyruvate	0.4	



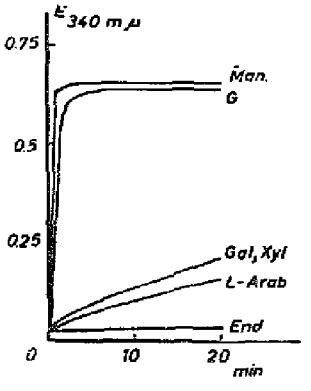


Fig. 2. Dehydrogenation of several aldoses by TPN in the presence of the particle-free supernatant of *A. suboxydans*. Content of the Beckman vessels, see text.

Fig. 1. Oxidation of several substrates by resting cells of *Acetobacter suboxydans*. Content of the Warburg vessels, see text. Substrates used: M (maltose), G (glucose), GA (gluconate), 5K (5-ketogluconate), 2K (2-ketogluconate), Et (ethanol), End (no substrate).

The oxidation of mannose proceeded at approximately the same rate as that of glucose. D-Galactose, D-xylose, L-arabinose were oxidized slowly (Fig. 2). D-Ribose and D-arabinose were not oxidized. The latter results are in agreement with our observation

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that this strain does not show acid production on yeast-extract (ribose or D-arabinose)–$CaCO_3$ slants. These dehydrogenases are obviously constitutive, since the cells had been grown on gluconate. These extracts also contain very active TPNH-linked reductases for 5- and 2-ketogluconates (Fig. 3 and Fig. 4). The activity of these

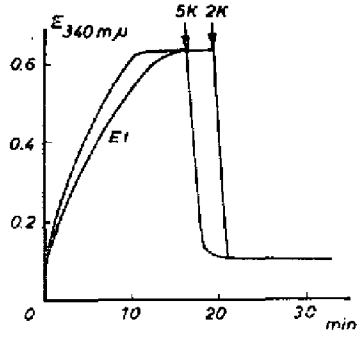

Fig. 3.

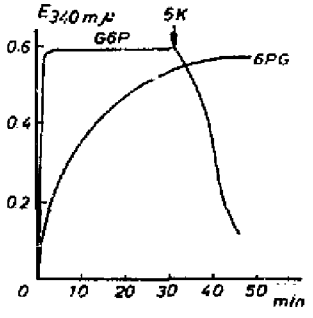

Fig. 4.

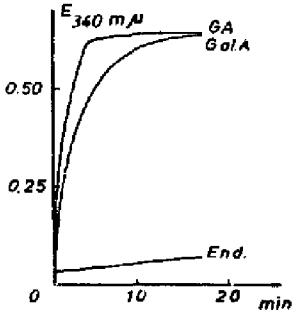

Fig. 5.

Fig. 3. A TPN-linked ethanol dehydrogenase and the 5K- and 2K-reductases in the particle-free supernatant of *A. suboxydans*. Content of the Beckman vessels, see text.

Fig. 4. TPN-linked G6P- and 6PG-dehydrogenases and the 5K-reductase in the particle-free supernatant of *A. suboxydans*. Content of the Beckman vessels, see text.

Fig. 5. Oxidation of gluconate and galactonate by TPN at pH 10 and a high substrate concentration with particle-free supernatant of *A. suboxydans*. Content of the Beckman vessels, see text.

enzymes is of the same order as the similar enzymes described in *Klebsiella* and *Escherichia*[14] and in *Corynebacterium helvolum*[16]. In the latter cases the end-product of the reaction was shown to be gluconate. A reaction with DPNH could not be demonstrated, for our preparations contained a very active DPNH-oxidase. No reductases were present for pyruvate, D-glucuronate, D-galacturonate and 5-keto-L-galactonate with TPNH.

The reactions:

$$\text{5-ketogluconate} + \text{TPNH} + \text{H}^+ \rightleftharpoons \text{gluconate} + \text{TPN}^+$$

and

$$\text{2-ketogluconate} + \text{TPNH} + \text{H}^+ \rightleftharpoons \text{gluconate} + \text{TPN}^+$$

are readily reversed at pH 10 and a high gluconate concentration. TPNH formation under these circumstances is shown in Fig. 5. Galactonate was also oxidized. Both hexonates were slowly oxidized in the presence of DPN. D-Gulonate, D-mannonate, D-arabonate, D-ribonate and D-xylonate were not oxidized with TPN.

In order to confirm the previous reactions and to detect the endproduct of the hexonate oxidation, Warburg-experiments were carried out in the presence of coenzymes and an auto-oxidizable carrier. N-methylphenazinium methyl sulfate was found to be superior to methylene blue for this purpose. Fig. 6 gives an example of such an experiment. In this way the coenzyme specificity could be checked. The rate of reaction with TPN was about 3 times as fast as with DPN. The oxidation of gluconate in the presence of TPN was about twice as fast as the galactonate oxidation under the same conditions. The endproducts of the oxidations were analyzed by paper chromatography. From D-gluconate both 2- and 5-ketogluconate had been formed. The oxidation of galactonate yielded only one spot, which had the same position and

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displayed the same colour as 2-ketogluconate, when sprayed with *o*-phenylenediamine. Thus probably 2-ketogalactonate had been produced.

(b) Kinases. It seemed quite possible that both 2- and 5-ketogluconate could be metabolized by way of kinases. Therefore experiments were set up, using the manometric method of COLOWICK AND KALCKAR, which in the absence of ATP can also demonstrate decarboxylase activity. Fig. 7 shows the results of such an experiment with the undialyzed particle-free supernatant. Both glucose and gluconate are phosphorylated to the corresponding phosphoric esters, since in each case 0.9 mole CO_2/mole substrate was liberated. However, with the ketogluconates the theoretical value of 1 CO_2 was exceeded: for 2-ketogluconate 1.2 mole CO_2 was obtained in this particular experiment and for 5-ketogluconate 1.9 mole CO_2/mole substrate. In the absence of ATP no CO_2 was formed, showing that decarboxylases for both ketohexonates were absent. The suggestion of FEWSTER, that 5-ketogluconate is first transformed into D-xylulose, could thus not be corroborated. The fact, that 2 moles CO_2 are liberated from 5-ketogluconate apparently suggests that, if 5-keto-6-phosphogluconate were formed, it would be decarboxylated with the formation of one extra mole CO_2. However, further investigation did not strengthen this view. When a dialyzed particle-free supernatant was used (Fig. 8), the rate of CO_2 evolution from 5-ketogluconate and ATP was markedly diminished. The rate of phosphorylation of glucose or gluconate was not influenced by dialysis. However, when the phosphorylation of ketogluconate was carried out in the presence of the catalytic amount of 0.3 μmole TPN, the original activity was restored. This result suggests then very strongly that 5-ketogluconate was not phosphorylated by a kinase, but converted by a more complex set of enzymes, requiring a catalytic amount of TPN, and an internal oxidation–reduction. This situation was analogous to other unpublished experiments, carried out previously in this laboratory[15] on the metabolism of 5-ketogluconate in *Klebsiella* and *Escherichia*, where the metabolism of this substrate proceeds by a combination of 5-ketoglucono-reductase, gluconokinase and 6PG-dehydrogenase. When these preparations of *Acetobacter suboxydans* were saturated with solid $(NH_4)_2SO_4$ all the proteins were precipitated. After centrifugation, the precipitate was dissolved in $M/100$ phosphate buffer pH 6.4 to restore the original volume and the solution was dialyzed to remove the $(NH_4)_2SO_4$. This preparation still contains a very active ATPase, but it scarcely phosphorylates 5-ketogluconate with ATP. Again when a catalytic amount of TPN is added, CO_2 evolution is restored to its original extent and 2 moles CO_2 are evolved (Fig. 9).

It is to be expected that 5-ketogluconate is thus ultimately converted into ribulose-5-phosphate and CO_2 and is oxidized through the hexose-monophosphate oxidative cycle. This could indeed be shown by the application of the cysteine–carbazole and the CyRI methods.

The appearance of an absorption maximum at 540 mμ with the cysteine–carbazole reaction shows that indeed a ketopentosephosphate is formed (Fig. 10). After 3 ½ h of incubation the O.D. at 540 mμ reached its maximum. Afterwards it diminished and the peak shifted towards the 560 mμ maximum, indicating the accumulation of fructose-6-phosphate. The CyRI showed that during the decomposition of 5-ketogluconate sedoheptulose-phosphate was slowly formed.

This mechanism, whereby 5K is metabolized only after a previous reduction offers an explanation for the phenomena observed by FEWSTER[12]. This author noticed that

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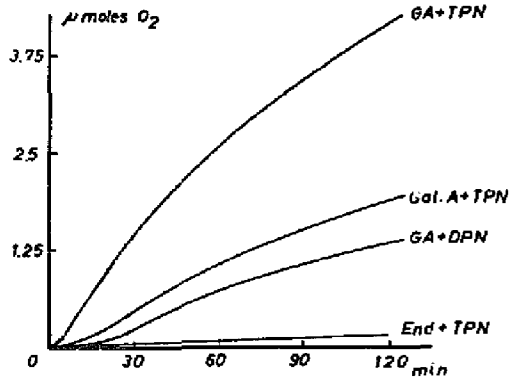

Fig. 6.

Fig. 6. Oxidation of gluconate and galactonate in the presence of TPN or DPN, N-methyl-phenazinium methylsulfate and the particle-free supernatant of *A. suboxydans*. Content of the Warburg vessels, see text.

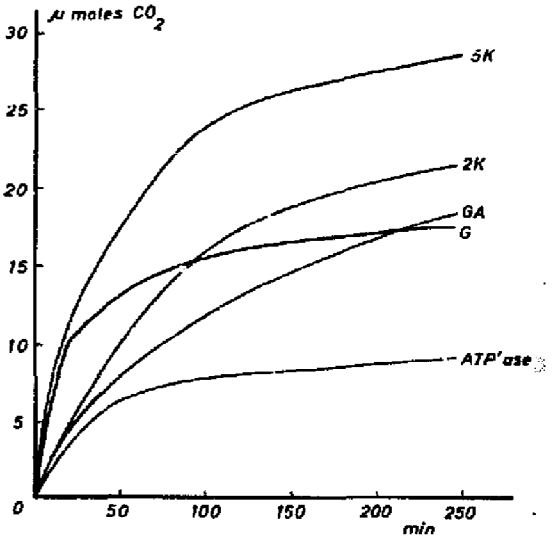

Fig. 7.

Fig. 7. The phosphorylation of glucose (G), gluconate (GA), 2- and 5-ketogluconate in the presence of ATP and the *undialyzed* particle-free supernatant of *A. suboxydans*. Content of the Warburg vessels, see text.

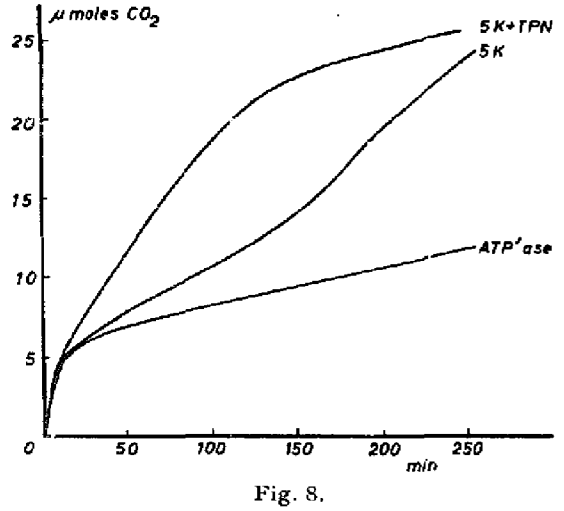

Fig. 8.

Fig. 8. The phosphorylation of 5-ketogluconate with ATP in the presence and absence of a catalytic amount of TPN by the *dialyzed* particle-free supernatant of *A. suboxydans*. Content of the Warburg vessels as for Fig. 7.

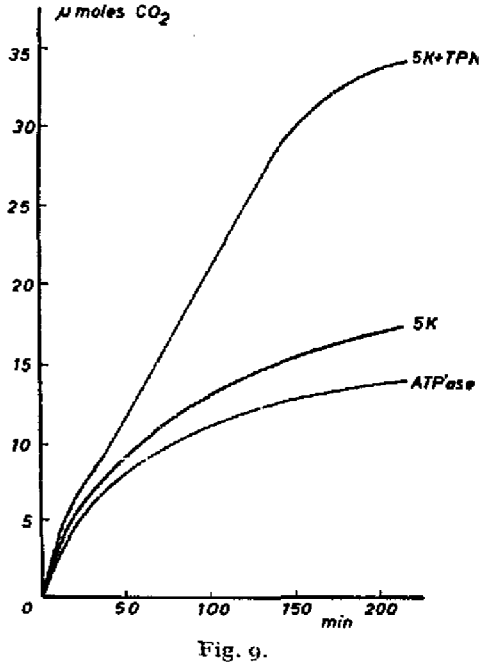

Fig. 9.

Fig. 9. Phosphorylation of 5-ketogluconate with ATP in the presence and absence of TPN. The enzyme preparation was obtained from the particle-free supernatant of *A. suboxydans*, by saturation with ammonium sulfate at pH 5–6, centrifugation of the proteins, redissolving them in the same amount of buffer and dialysis. Content of the Warburg vessels as before.

resting cells of his strain oxidized 5K only when a trace of some other oxidizable substance was present (either from a slightly decomposed 5K solution, obtained by standing several days at room temperature, or from the endogenous substrates in the cells). They obviously use these substances as a source of TPNH to initiate 5K respiration. Thoroughly washed cells of our strain oxidized 5K less rapidly; fresh or aged solutions of 5K were oxidized at the same rate. Apparently our strain still contained enough endogenous substrates to spark the 5K oxidation.

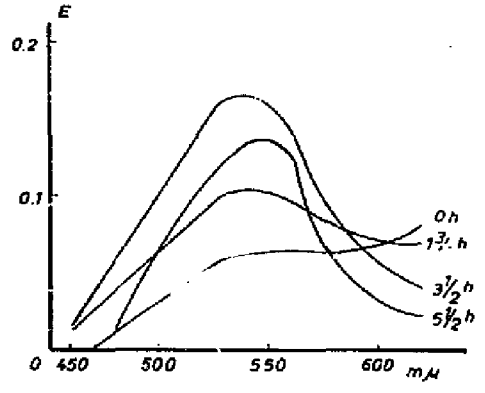

Fig. 10.

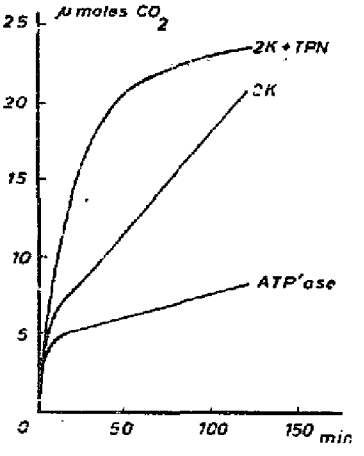

Fig. 11.

Fig. 10. The formation of ketopentose phosphates and fructose phosphate during the phosphorylation of 5-ketogluconate with ATP by the dialyzed particle-free supernatant of *A. suboxydans* in the presence of TPN. Cysteine–carbazole reaction. Composition of the reaction mixture, see text.

Fig. 11. The phosphorylation of 2-ketogluconate with ATP in the presence and absence of a catalytic amount of TPN by the *dialyzed* particle-free supernatant of *A. suboxydans*. Content of the Warburg vessels, see text.

It is very probable, that the mechanism of 2-ketogluconate follows the same pattern as that of 5-ketogluconate. Dialyzed extracts are still able to phosphorylate 2-ketogluconate at a considerable rate in the presence of ATP. Here also the phosphorylation in the presence of a catalytic amount of TPN proceeds much faster. In both cases approximately 1.5 to 1.6 moles CO_2/mole substrate are formed (Fig. 11). It cannot be excluded that part of the 2-ketogluconate is phosphorylated by a 2-ketogluconokinase as described in *Aerobacter cloacae*[23,24,25].

3. *Oxidations with particulate enzymes*

Acetobacter suboxydans contains, moreover, a third enzyme for the oxidation of gluconate: a particle-linked, possibly cytochrome-linked gluconate oxidase. The particles oxidize the substrate with an O_2 uptake of 0.5 mole O_2/mole substrate. By paper chromatography and colorimetric methods it could be shown that the end product consisted solely of 2-ketogluconate. No trace of 5-ketogluconate could be detected. This oxidation is independent of added TPN or DPN.

Fig. 12 shows the pH optimum to be around 5. D-Galactonate is oxidized at 1/10, D-gulonate and D-mannonate at about 1/20 of the rate of gluconate. Maltobionate,

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lactobionate, D-ribonate, D-arabonate, galacturonate and glucuronate are not oxidized. The packed particles after centrifugation are distinctly red. With a hand spectroscope the following cytochrome bands can be seen: at 555 mμ a very strong

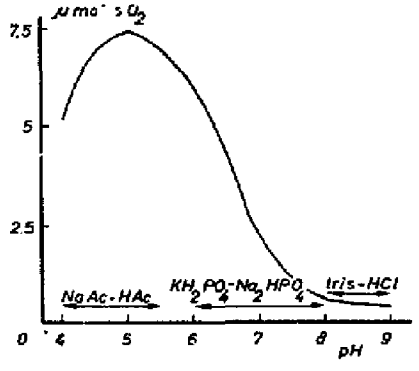

Fig. 12.

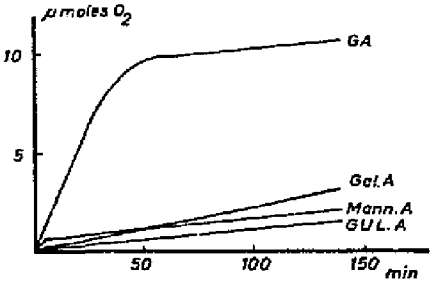

Fig. 13.

Fig. 12. Oxidation rate of gluconate by the particles of *A. suboxydans* as a function of the pH. The O_2 uptake after 25 min is plotted.

Fig. 13. The oxidation of gluconate (GA), galactonate (GalA), mannonate (Mann. A) and gulonate (GUL. A) by the particles of *A. suboxydans*. Content of the Warburg vessels, see text.

band, between 520–525 mμ a strong one, between 560–570 and 528-534 weak ones and a very weak band at about 515 mμ. When a suspension of particles is shaken in air, it becomes practically colourless and the cytochrome bands disappear. When a

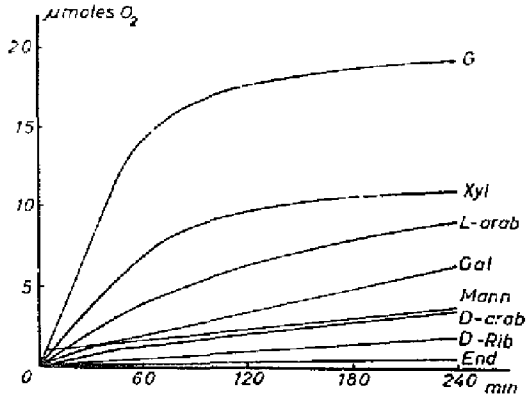

Fig. 14.

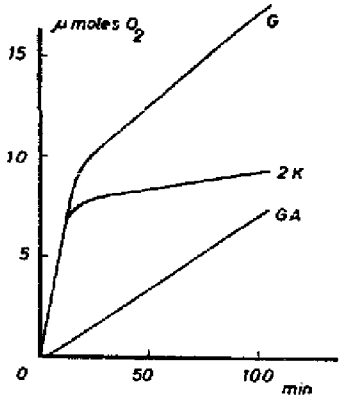

Fig. 15.

Fig. 14. The oxidation of several hexoses and pentoses by the particles of *A. suboxydans*. Glucose (G), galactose (Gal), mannose (Mann), xylose (Xyl), L-arabinose (L-arab), D-arabinose (D-arab) and ribose (D-rib).

Fig. 15. The oxidation of glucose (G), gluconate (GA) and 2-ketogluconate (2K) by particles of *A. melanogenum*. Content of the Warburg vessels, see text.

few μmoles of gluconate are added, the suspension at once becomes reddish and the cytochrome bands are again visible. These particles are also able to oxidize D-mannitol with an uptake of 0.5 mole O_2 and glucose, D- and L-lactate with an uptake of 1 mole O_2/mole substrate. D-Xylose and L-arabinose are oxidized with the uptake of

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0.5 mole O_2 and the formation of a lactone and lactonizable acid. Galactose and D-arabinose are slowly oxidized, mannose and ribose scarcely at all (Fig. 14).

The acid formation obtained by growing cultures on sugar–yeast extract–$CaCO_3$ slants in the case of mannose is mainly due to the soluble TPN-linked dehydrogenase, while with L-arabinose and D-xylose the particle-linked oxidases are responsible.

Gluconate oxidases have been described in *Pseudomonas aeruginosa*[26] and in *Pseudomonas fluorescens*[27]. In both cases the enzyme were particle-linked. The properties of these enzymes have been studied after they had been detached from the particles and therefore it cannot be determined whether these 3 enzymes are identical or not.

4. *Enzyme systems in* Acetobacter melanogenum

We also made a comparison between the metabolism of the hexonic and keto-hexonic acids in our *Acetobacter suboxydans* and that of a strain of *Acetobacter melanogenum*, which we obtained by the courtesy of Prof. Dr. T. O. WIKEN, Delft. Before the comparison was carried out, this strain had been maintained on yeast extract–glucose–$CaCO_3$ slants for one year. At first it produced quite rapidly the brown coloration characteristic for *Acetobacter melanogenum*, which was shown by KATZNELSON, TANENBAUM AND TATUM[7] to be due to the formation of 2,5-diketo-gluconate from 2-ketogluconate. At that time it produced only few crystals of Ca-5-ketogluconate. During this period the properties of the strain changed. At present it produces large amounts of Ca-5-ketogluconate and sometimes it is necessary to incubate the slants for 7 days before a weak brown coloration can be observed. Comparable changes in the production of ketogluconate have been reported by BERNHAUER AND GÖRLICH[2] for *Acetobacter gluconicum* and by KULKA AND WALKER[28] for several other *Acetobacters*. This may also be the reason why FODA AND VAUGHN[29] reported that *Acetobacter melanogenum* produces only 5-ketogluconate, while KATZNELSON *et al.* reported at the same time that *Acetobacter melanogenum* produced 2,5-diketogluconate and that this was the reason for the brown coloration.

Since the mechanism of the metabolism of 2- and 5-ketogluconate in *Acetobacter suboxydans* had been worked out, it was thought interesting to give an enzymic explanation of these changes. Unfortunately, however, the enzymic arrangement before the change could not be established, but it can safely be assumed that originally there was only a weak 5-ketogluconoreductase and an active system for the production of 2-ketogluconate. This strain was always grown on the glucose medium. Resting cells oxidized gluconate with an average O_2 uptake of 0.6 mole/mole substrate.

Suspensions of both young and old cells of the used strain oxidized 2-ketogluconate with an O_2 consumption of 0.5 mole/mole substrate. The further oxidation of 2,5-diketogluconate to α-ketoglutaric acid, described by DATTA AND KATZNELSON[8] is thus absent in our strain. 5-Ketogluconate is not oxidized.

The results with resting cells already give a partial explanation of the lack of brown colour formation by this changed strain; although 2-ketogluconate can still be oxidized rapidly to 2,5-diketogluconate, yet gluconate is oxidized mainly to 5-keto-gluconate. Cell-free extracts were prepared and separated into a dark brown particulate fraction and a particle-free supernatant. These particles oxidized glucose rapidly with an O_2 consumption of 0.5 mole/mole substrate, then the rate of O_2 uptake dropped and became identical to that of gluconate oxidation. 2-Ketogluconate is oxidized with an

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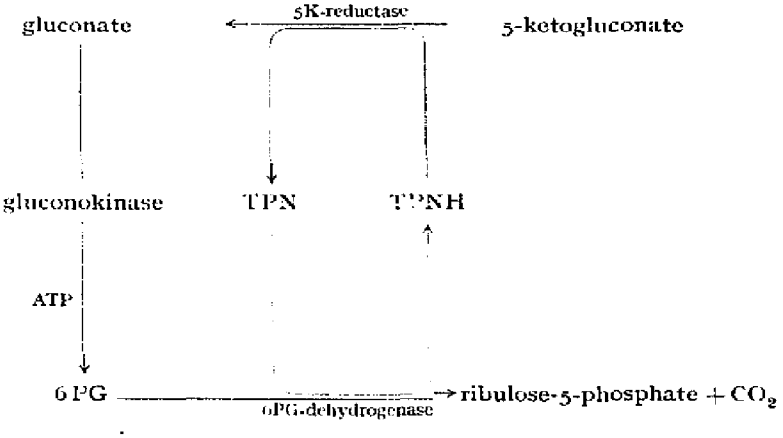
uptake of 0.4 mole O_2/mole substrate (Fig. 15). The particle-free supernatant had no TPN-linked 2-ketogluconoreductase. It contained, however, a very active 5-ketogluconoreductase. These enzymic data explain the physiological behaviour and the change that occurred in the strain: obviously the enzymes for the production of 2-ketogluconate from gluconate in this strain have lost most of their activity and therefore not enough 2-ketogluconate, and from this 2,5-diketogluconate could be formed for the production of the brown colour. The result is, that gluconate is oxidized mainly by the 5-ketogluconoreductase. These extracts do not contain a gluconokinase. This finding explains why 5-ketogluconate is not oxidized by resting *Acetobacter melanogenum* cells and corroborates the reaction sequence postulated above, according to which gluconokinase is a member of the set of enzymes for the metabolism of 5-ketogluconate.

DISCUSSION

The principal feature of the catabolism of ketohexonic acids by micro-organisms has also been confirmed in the genus of *Acetobacter*. It has previously been shown that members of the family of the Enterobacteriaceae, of Bacilli, of Corynebacteria, some yeasts and some molds metabolize ketohexonic acids only after a previous reduction. Also from this investigation it appears that 2- and 5-ketogluconate are not on the main pathway of sugar metabolism in the genus *Acetobacter*.

The catabolism of 5-ketogluconate is represented by the following scheme:



The formation of 2,5-diketogluconate appears to be the only exception to this rule. The liberal formation of the two ketohexonates in this genus is only a side path and here also they have to be reduced to gluconate, before they can enter the hexose–monophosphate oxidative cycle.

ACKNOWLEDGEMENTS

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STUDIES ON THE ELECTRON TRANSPORT SYSTEM

XXIII. COENZYME Q OXIDASE

YOUSSEF HATEFI* with the technical assistance of FELIPE QUIROZ-PEREZ

Institute for Enzyme Research, University of Wisconsin, Madison, Wisc. (U.S.A.)

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SUMMARY

The preparation and properties of a submitochondrial enzyme system containing predominantly cytochrome *a* have been described. This system is capable of catalyzing the oxidation of reduced coenzyme Q by molecular oxygen. This particulate enzyme requires the presence of both cytochrome *c* and a lipoprotein of mitochondrial origin in order to catalyze the above reaction. The results presented in this communication suggest that coenzyme Q is situated in the aforementioned lipoprotein between cytochromes *b* and *c*.

The abbreviations used in the text are as follows: DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; ADP, adenosine diphosphate; Tris, Tris/hydroxymethyl)aminomethane; and QH_2, the reduced form of coenzyme Q. Coenzyme Q_{10}[12] was the homologue used throughout this investigation.

* Postdoctoral trainee of the University of Wisconsin, Institute for Enzyme Research.

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