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Physiology of *Gluconobacter oxydans* during dihydroxyacetone production from glycerol

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Abstract Investigations into physiological aspects of glycerol conversion to dihydroxyacetone (DHA) by *Gluconobacter oxydans* ATCC 621 were made. The activity levels of the enzymes involved in the three catabolic pathways previously known and the effects of specific inhibitors and uncoupling agents on cellular development, DHA synthesis, and cellular respiratory activity were determined. It was established that only two catabolic pathways are involved in glycerol dissimilation by this micro-organism. The only enzyme responsible for DHA production is membrane-bound glycerol dehydrogenase, which employs oxygen as the final acceptor of reduced equivalents without NADH mediation. The ketone is directly released into the culture broth. As the glycolytic and carboxylic acid pathways are absent, the pathway provided by the membrane-bound enzyme is indispensable for the energy requirements of *G. oxydans*. The cytoplasmic pathway, which begins by phosphorylation of glycerol followed by a dehydrogenation to dihydroxyacetone phosphate, allows growth of the bacterium. At the same time, the substrate transport mode was characterized as facilitated diffusion using radioactive [1(3)-^{3}H]-glycerol. Concerning the DHA inhibition of microbial activity, the enzymatic study of the membrane-bound glycerol dehydrogenase showed the enzymatic origin of this phenomenon: a 50% decrease of the enzyme activity was observed in the presence of 576 mM DHA. The decrease in the rate of penetration of glycerol into cells in the presence of DHA indicates that growth inhibition is essentially due to the high inhibition exerted by the ketone on the substrate transport system.

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Introduction

Dihydroxyacetone (DHA) is a compound commonly used for pharmaceutical purposes and chemical syntheses. It is produced industrially during fermentation of glycerol by *Gluconobacter oxydans*. The main characteristic of this micro-organism, which belongs to the family of acetic acid bacteria, is the high oxidative power of its enzymatic equipment on sugars and polyhydric substrates (De Ley and Dochy 1960; De Ley and Kersters 1964).

Scientific papers describe three possible pathways involved in glycerol conversion by *G. oxydans* (King and Cheldelin 1952a, b; Hauge et al. 1955a, b; Ameyama et al. 1985). Glycerol can be directly oxidized into DHA by a glycerol dehydrogenase present in the cell membrane. The enzyme activity is uncharacteristic owing to the fact that it is NAD-independent. A respiratory electron transfer chain using ubiquinone and cytochrome *o* allows oxygen (O_2) to accept the electrons formed during this oxidation of glycerol (Hauge et al. 1955a; Yamada et al. 1969; Ameyama et al. 1981, 1985).

In the cytoplasm, glycerol is either converted to dihydroxyacetone phosphate (DHA-P) by phosphorylation (glycerokinase) followed by dehydrogenation to DHA-P [glycerol-3-phosphate (G-3-P) dehydrogenase] or is converted to DHA by dehydrogenation (glycerol dehydrogenase) followed by phosphorylation (DHA kinase) to DHA-P. Dehydrogenation steps are

linked to the presence of the cofactor NAD and DHA-P produced is then catabolized by the pentose-phosphate pathway (King and Cheldelin 1952a, b; Hauge et al. 1955a, b).

The involvement of the different metabolic pathways during DHA synthesis and growth phenomena, the localization of the DHA production and the mode of penetration of glycerol into the cells are essential factors for a complete understanding of *G. oxydans* glycerol catabolism. As these factors had not been already defined, our first objective was to investigate the respective roles played by the enzymes of the plasma membrane and the cytoplasmic enzyme system during the different phases of cellular activity.

Recent work (Bories et al. 1991; Claret et al. 1992, 1993) on glycerol conversion by *G. oxydans* show that this fermentation is subject to inhibition phenomena:

1. A high initial glycerol content exerts an inhibitory effect on the growth rate and on the rate of DHA production.

2. An increase in DHA concentration in the culture medium causes a greater inhibition: a decrease in the kinetics of the microbial activities, and particularly of bacterial growth first occurs, then cellular development and DHA synthesis cease completely.

The second objective of this paper was to provide an explanation of the mechanisms involved in this DHA inhibition.

Materials and methods

Micro-organisms and culture conditions

The microbial strain used, *G. oxydans* ATCC 621 (American Type Culture Collection, Rockville, M., USA) was cultivated in a liquid medium containing 10 $g l^{-1}$ of yeast extract and glycerol (20 to 100 $g l^{-1}$).

Bacterial cultures were performed either in 2-erlenmeyer flasks containing 200 ml medium (initial pH 7.0) and placed on an orbital shaker (LSL Biolafitte, Poissy, France) (130 rpm) in a thermostated room (28°C), or in 2-LSL Biolafitte (Poissy, France) reactors containing 1.5 of culture medium with the pH and temperature adjusted to 6.0 and 28°C, respectively. Agitation and aeration were controlled at 800 rpm and 1 vvm, respectively, as previously described (Bories et al. 1991).

Analytical methods

Biomass dry weight was obtained by centrifuging culture aliquots (Beckman centrifuge, Palo Alto, Calif., USA), washing twice with distilled water and drying at 105°C until a constant weight was reached. During cultures, biomass formation was evaluated by measurement of the optical density at 620 nm with a Varian spectrophotometer (Springvale, Australia) (Claret et al. 1992).

Organic compounds (glycerol, DHA, succinate) were determined by HPLC after filtration and appropriate dilution of samples using an Aminex Biorad HPX-87 H column (Richmond, Calif., USA) thermostated at 40°C and eluted by a continuous flow of 0.005 M H_2SO_4 eluant (0.7 ml min^{-1}). Glycerol and DHA were detected with a Waters refractometer (Milford, New England, USA) and succinate by an Shimadzu UV spectrophotometer (Kyoto, Japan). The concentrations were calculated by a Shimadzu integrator (Kyoto, Japan).

Preparation of cells and cell-free extracts

Cultures were harvested after 12 h of incubation (growing cells) or after 48 h of incubation (resting cells). Cells were centrifuged (5°C, 10 min, 50 000 *g*) and washed in a phosphate buffer (with a concentration factor if necessary), or in a nutritive medium either with or without the addition of an uncoupling agent or inhibitor.

For preparation of cell-free extracts, the micro-organisms were concentrated 20-fold in phosphate buffer and disrupted by mechanical agitation in the presence of glass beads (diameter 0.1 mm) at a bead volume/cellular suspension volume ratio of 2 for 10 min at 5°C. The suspension was immediately centrifuged (Beckman centrifuge, 50 000 *g*) and two fractions were harvested: (1) the supernatant fraction, which contained all the cytoplasmic enzymes and some membrane-bound solubilized enzymes; (2) the precipitable fraction, which contained the membrane-bound enzymes. The fraction aliquots were conserved at − 20°C.

Enzyme assays

The cytoplasmic dehydrogenase activities [glycerol dehydrogenase activity (Kersters and De Ley 1966), G-3-P dehydrogenase activity (Battle and Collon 1979)] were measured spectrophotometrically by following the substrate-dependent formation of NADH at 340 nm (Varian spectrophotometer, Springvale, Australia).

Kinase activities were indirectly measured as previously described by Battle and Collon (1979).

Membrane-bound glycerol dehydrogenase activity was determined using 2,6-dichlorophenolindophenol (2,6-DCIP) and phenazine methosulphate as final electron acceptors. 2,6-DCIP reduction was measured spectrophotometrically at 590 nm.

Determination of the effect of uncoupling agents on microbial activity

For determination of the effect of uncoupling or inhibitory agents on microbial activity, cells were suspended in glycerol nutritive medium containing the agents at the required concentration. Growth and DHA synthesis were evaluated by measuring the changes in optical density and the DHA concentration.

O_2 consumption was determined by measuring the decrease in O_2 concentration in a closed cell using a Clark electrode.

Determination of intracellular concentrations

To determine the intracellular concentration of glycerol and DHA, a tracer, xylose, was added to the culture aliquots (final concentration, 20 $g l^{-1}$) and the bacterial suspension was centrifuged (Beckman centrifuge, 50 000 *g*). Glycerol, DHA and xylose were analysed from the supernatant. The cells were washed three-fold and concentred 20-fold in 0.1 M phosphate buffer. Cell-free extracts were then prepared and the compounds in the cytoplasmic fraction were determined.

The cell volume was evaluated by a method using the study of the dilution of a marker in the interstice volume as described by Reus et al. (1979). The used marker was the Cl^- ion, and its concentration

was determined by ionic chromatography (DX-100 Dionex ion chromatograph, Sunnyvale, Calif., USA).

Determination of the rate of glycerol penetration

A 20-µl aliquot of bacterial suspension was mixed with 20 µl glycerol solution containing [1(3) – ^{3}H]-glycerol (glycerol concentration range, 50 mM to 2M). The assays were performed with a constant amount of labelled glycerol. The glycerol solution specific radioactivity ranged from 0.37 to 13.8 MBq · mmol^{-1}.

After a 3-min incubation at 28°C, uptake was stopped by dilution with 10 ml ice-cold phosphate buffer (0.1 M, pH 7.0) and filtration (Whatman glass microfibre filters type GF/C; Whatman cellulose nitrate membrane filters, pore size 0.2 µm, Maidstone, Kent, UK). The cells collected on the filters were washed with 10 ml ice-cold buffer, dried and assayed for radioactivity (Betamatic V scintillation liquid counter, Kontron Instrument, Switzerland). Uptake was directly proportional to the time for the first 7 min of incubation.

Results

Metabolism of glycerol by *Gluconobacter oxydans*

The membrane-bound glycerol dehydrogenase, cytoplasmic glycerol dehydrogenase, glycerokinase, DHA kinase, and G-3-P dehydrogenase specific activities were determined from *G. oxydans* cells harvested during the growth phase of a culture performed with an initial glycerol content of 51 g l^{-1} (Table 1).

When *G. oxydans* was cultivated on a glycerol medium, only membrane-bound glycerol dehydrogenase, glycerokinase and G-3-P dehydrogenase activities were detectable. Neither cytoplasmic glycerol dehydrogenase nor DHA kinase activities were found.

To specify the way in which the different pathways of glycerol catabolism work (especially the mode of regeneration of reduced co-enzymes), the effect of inhibitory compounds on the microbial growth, on the specific rate of DHA production, and on the specific rate of O_2 consumption was analysed. Different kinds of inhibitory compounds were used: specific inhibitors of enzymes involved in the electron transfer of the respiratory chain [potassium cyanide (KCN), antimycin A], uncoupling agents (gramicidin) and the more complex compound sodium azide (NaN_3) (Table 2).

Table 1. Comparison of the level of activity of the main enzyme involved in glycerol catabolism contained by *Gluconobacter oxydans* cells cultivated on a glycerol medium (initial glycerol concentration = 51 g l^{-1})

Studied enzyme	Enzymatic activity (kat mg^{-1} protein)
Cytoplasmic glycerol dehydrogenase	nd
Membrane glycerol dehydrogenase	$7.03\ 10^{-9} \pm 0.38\ 10^{-10}$ [a]
DHA kinase	nd
Glycerokinase	$2.25\ 10^{-9} \pm 0.13\ 10^{-10}$ [a]
Glycerol-3-phosphate dehydrogenase	$0.81\ 10^{-9} \pm 0.03\ 10^{-10}$ [a]

The enzymatic determinations were made after 12 h of cultivation: nd, not detected; DHA, dihydroxyacetone

[a] Mean values and standard deviations of four experiments for each determination

The effect of inhibitors on the electron transfer chain depends on the specificity of the inhibitory compound. In the presence of KCN, which inhibits the cytochrome oxidases (type *c* and *o*) and consequently oxidative phosphorylation, growth, DHA synthesis, and O_2 consumption were blocked. Antimycin A acts specifically on the cytochrome *c* oxidase activity and so has an effect solely on the transfer of electrons through the classical respiratory chain used by aerobic micro-organisms for the regeneration of reduced co-enzymes (NADH + H$^+$). This compound did not inhibit bacterial growth or DHA formation during glycerol catabolism by *G. oxydans*. At the same time, it was observed that all the uncoupling agents tested totally inhibited development (Table 2): the production of ATP, necessary for growth, was not ensured. They increased the rate of DHA synthesis but differed in their effect on O_2 consumption. In the presence of gramicidin, the rate of O_2 consumption by growing cells increased to a lesser extent than the rate of DHA synthesis (Table 3). In this case, production of succinate was observed. This synthesis was attributed to fumarate reduction by a succinate dehydrogenase. In contrast, NaN_3 decreased the respiratory activity, DHA production rate was increased slightly and succinate production was noticed.

Finally, the localization of DHA formation was examined. As DHA is synthesized by an enzyme localized on one face of the plasma membrane, it is possible for it to be produced either inside or outside the cell. The intracellular and extracellular concentrations of DHA were compared in cells harvested at various stages of the culture (Table 4). These results, established by means of HPLC determinations, were verified by experiments using [^{13}C] glycerol and nuclear magnetic resonance (NMR) analysis. No DHA was detected in the cytoplasmic fraction at any time of the experiment; the DHA, a toxic compound formed by oxidation of glycerol, is produced directly into the culture medium or very efficiently excreted from the cytoplasm.

Physiology of inhibition by DHA

Inhibition of the DHA synthesis. In *G. oxydans*, glycerol oxidation into DHA is achieved in one step, by a membrane-bound glycerol dehydrogenase. Thus, it seems likely that the inhibition of the ketone production by DHA is of enzymatic origin. To test this hypothesis, the

Table 2. Effect of uncoupling and inhibitory compounds on the microbial activity of *G. oxydans*

Assay conditions	Microbial growth		Resting cells		
	($\mu\ h^{-1}$)	Maximal biomass $g\,l^{-1}$	q_{O2}[a] (mmol $O_2\ g^{-1}$ dry weight h^{-1})	q_{DHA}[a] (mmol g^{-1} dry weight h^{-1})	Succinate production[a,b] (mmol g^{-1} dry weight h^{-1})
Standard conditions	0.38	1.8	14.5 ± 0.80	29.7	—
KCN (1 mM)	0.00	0.09	1.7 ± 0.09	0.0	—
Antimycin A (20 µM)	0.35	1.8	nd	28.6 ± 1.63	—
NaN_3 (0.5 mM)	0.00	0.07	7.7 ± 0.43	33.1 ± 1.54	14.2 ± 0.33
Gramicidin (1 mM)	0.06	0.7	13.9 ± 0.77	nd	nd

µ, Specific growth rate; q_{O2}, specific rate of O_2 consumption; q_{DHA}, specific rate of DHA production; nd, not determined

[a] Means values and standard deviation of three experiments for each determination

[b] No succinate production

Table 3. Effect of gramicidin on the microbial activity of growing cells of *G. oxydans*

Assay conditions	q_{O2} (mmol $O_2\ g^{-1}$ dry weight h^{-1})	q_{DHA} (mmol DHA g^{-1} dry weight h^{-1})	Succinate production (mmol g^{-1} dry weight h^{-1})
Standard conditions	17.5 ± 0.99	29.5 ± 1.25	—
Gramicidin (1 mM)	20.6 ± 1.15	46.6 ± 1.94	7.1 ± 0.46

Means values and standard deviation of three experiments for each determination

Table 4. Comparison between intracellular and extracellular concentrations of glycerol and DHA during *G. oxydans* culture on glycerol

Experimental conditions	concentration ($g\,l^{-1}$)	
	Intracellular	Extracellular
5 h of cultivation		
Glycerol	47	45
DHA	< 0.1	2
Xylose	< 0.5	20
12 h of cultivation		
Glycerol	20	18.5
DHA	< 0.5	29
Xylose	< 0.1	19.9
24 h of cultivation		
Glycerol	0	0
DHA	< 0.5	47
Xylose	< 0.5	20

activity of the membrane-bound glycerol dehydrogenase was measured in experiments using increasing concentrations of DHA (0, 0.2, 0.4, 0.6, 1.0 and 2.0 M) (Fig. 1).

The glycerol dehydrogenase specific activity detected in the presence of 576 mM DHA represented only 50% of the reference activity, obtained without initial addition of DHA. An increase in the initial DHA concentration induced a strong decrease in the specific activity of the membrane-bound enzyme.

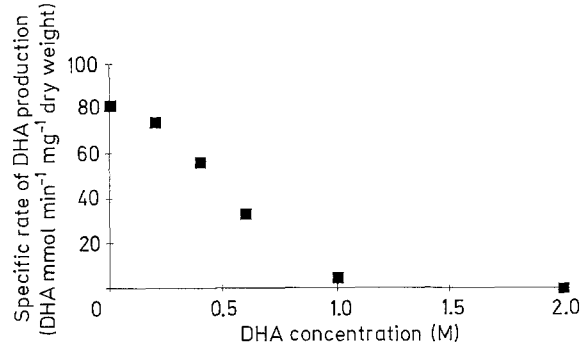

Fig. 1. Effect of dihydroxyacetone (DHA) on membrane-bound glycerol dehydrogenase activity

Growth inhibition by DHA. Firstly, we tried to characterize the mechanism by which glycerol penetrates into *G. oxydans* cells. The initial rates of glycerol penetration were determined using the incorporation of [1(3) – ^{3}H]-glycerol into the cells (Fig. 2). Micro-organisms and labelled glycerol were incubated in the presence of increasing concentrations of extracellular glycerol and varying the conditions as follows:

1. standard conditions: 0.1 M phosphate buffer, pH 7.0.

2. addition of uncoupling agent: 0.1 M phosphate buffer, pH 7.0, 0.5 mM NaN_3 and 0.5 mM 2,4-DNP.

3. addition of inhibitor of the respiratory chain: 0.1 M phosphate buffer, pH 7.0, 1 mM KCN.

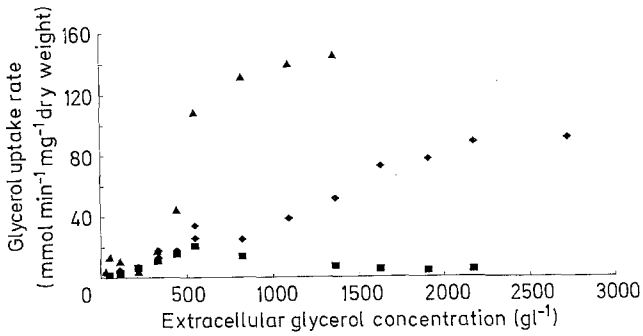

Fig. 2. Changes in the specific rate of glycerol uptake by *Gluconobacter oxydans* cells with different extracellular substrate concentrations performed under standard conditions (incubations of cells in phosphate buffer containing glycerol, (♦), in the presence of 1 mM KCN (▲) and in the presents of uncoupling agents 0.5 mM 2,4-dinitrophenol (2,4-DNP) and 0.5 mM sodium azide, (■)

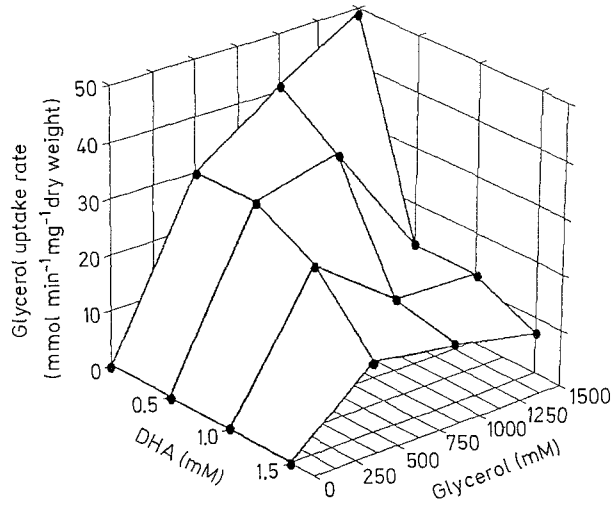

Fig. 3. Changes in the glycerol penetration rate in *G. oxydans* with different concentrations of glycerol and DHA

Only in the presence of KCN was a true glycerol penetration into the cells observed. Evidently both cytoplasmic glycerol catabolism and substrate conversion by the plasmic membrane-bound enzyme are totally inhibited by KCN. Under these incubation conditions and up to 500 mM external glycerol concentration, the initial glycerol uptake rate was directly proportional to the concentration of extracellular substrate. With an external glycerol concentration higher than 500 mM, the rate of substrate penetration into cells became constant. Thus, in *G. oxydans*, glycerol transport seems to be a saturable process.

Under standard conditions, without addition of inhibitory or uncoupling agents, glycerol was both metabolized at the membrane and at the cytoplasmic levels. In this case, the specific initial rate of glycerol uptake was lower than the rate observed in the presence of KCN. Therefore, the transport system must be inhibited by an intermediate or an end-product of glycerol catabolism.

In the presence of uncoupling agents, the specific rate of glycerol uptake decreased for extracellular concentrations of glycerol above 500 mM. Under these conditions, the inhibition phenomenon was much higher. It was previously shown that when the cells are incubated in presence of 2,4-dinitrophenol (2,4-DNP) and NaN_3 the cytoplasmic glycerol catabolism is entirely blocked, but the ability of the cell membrane enzymatic equipment to convert substrate into DHA is improved. Thus, it seems likely that the inhibition of the substrate penetration into the cell is due to the main product of the membrane-bound enzyme: DHA

Two further sets of experiments were performed to verify the above assumption. Applying the same conditions as the previous experiment, the quantity of DHA produced by the cells in 3 min (incubation time used for the measurement of initial specific rates) was evaluated and compared (data not shown). KCN totally inhibited the DHA synthesis, whereas under standard conditions or in the presence of uncoupling agents, 0.22 and 0.4 mmol DHA mg^{-1} dry weight were produced respectively in 3 min for a glycerol extracellular concentration of 500 mM.

Finally, DHA was added at different concentrations and the specific rates of glycerol uptake by *G. oxydans* were measured. The concentrations of DHA used, between 0 mM and 1.5 mM, were chosen in consideration of the previous results (Fig. 3).

The rate of substrate penetration into the cells decreased as the concentration of extracellular DHA (produced or added) rose, irrespective of the concentration of glycerol used. DHA at a concentration of 1.5 mM (0.13 gl^{-1}) inhibited the rate of glycerol uptake by 60% and 90% respectively, for glycerol external concentrations of 500 and 1500 mM. Evidently, the presence of DHA has a marked effect on the mechanism of glycerol transport.

Discussion

This research removes the ambiguity concerning the pathways involved in glycerol catabolism by *G. oxydans*, determines their implication in the growth phenomenon and in the DHA synthesis process and specifies their way of working. The respective roles of the three different catabolic pathways described in the literature for glycerol dissimilation by acetic acid bacteria, in DHA synthesis and the growth process were studied: with this objective, the activities of the main enzymes involved in the first steps of the named pathways were assayed. Thus it was demonstrated that there are only two pathways involved in glycerol catabolism by *G. oxydans*:

1. Glycerol assimilation by phosphorylation into G-3-P followed by dehydrogenation of G-3-P into DHA-P. This conversion occurs in the cytoplasm and there is no DHA formation in this cellular compartment. DHA-P was then catabolized by means of the pentose-phosphate pathway, in order to ensure microbial growth.

2. Glycerol dissimilation achieved by direct oxidation using a membrane-bound glycerol dehydrogenase, which appears to be the only catabolic enzyme responsible for DHA synthesis.

The above results are quite different from those obtained in previous work. In fact, Hauge et al. (1955a) reported the presence of DHA kinase and glycerol dehydrogenase activities in cell-free extracts of *G. oxydans*. These authors did not use specific methods for enzymatic determinations nor did they differentiate between cytoplasmic and membrane-bound enzymes. They could have mistaken membrane-bound dehydrogenase activity for cytoplasmic dehydrogenase activity. Moreover, DHA phosphorylation, which was attributed to DHA kinase action, could have been achieved by a glycerol kinase, this enzyme having a low substrate specificity. Analysis of the inhibitory effects of the enzyme inhibitors or uncoupling agents specifies the different ways of working of the two metabolic pathways for glycerol dissimilation, as summarized in Fig. 4.

The cytoplasmic pathway, which ensures the microbial growth, is ATP-dependent and uses NAD-dependent dehydrogenases. The membrane-bound pathway, which directly oxidises glycerol into DHA is ATP- and NAD-dependent. As the glycolysis pathway and the carboxylic acid cycle, which normally ensure energy synthesis are absent in *G. oxydans* cells, the membrane-bound oxidation of glycerol is essential for providing the energy necessary for cell growth, This fact explains the earlier observation that the membrane-bound enzyme is responsible for most of the glycerol converted. This glycerol conversion leads to an extracellular production of DHA and uses O_2 as the final acceptor of electrons and reduced equivalents by means of ubiquinone and cytochrome *o*, this special chain of electron transfer being coupled to ADP phosphorylation.

In the absence of metabolic inhibitors, electrons are transferred from the substrate to O_2, which plays the role of final acceptor, through a particular respiratory chain, composed of ubiquinone, cytochrome *o* and specific oxidases, previously described by Ameyama et al. (1987) and by Matsushita et al. (1987). This chain is coupled to ADP phosphorylation into ATP: when the classical system of oxidative phosphorylation, which is involved in the regeneration of the reduced co-enzymes, is specifically inhibited (in the presence of antimycin A), this ensures the synthesis of energy and allows growth to occur.

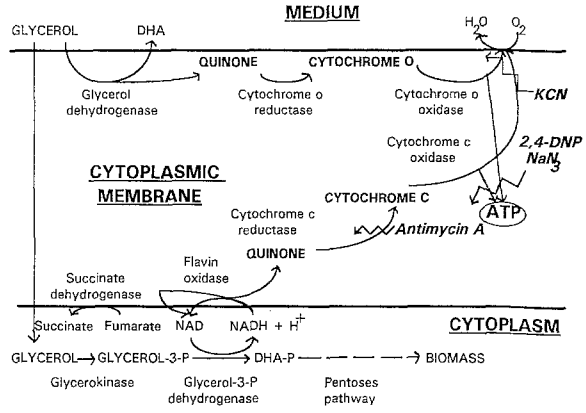

Fig. 4. Metabolic pathways used for glycerol conversion by *G. oxydans*: DHA-P, dihydroxyacetone phosphate

In the presence of uncoupling agents or NaN_3, inhibition of ATPase creates a pronounced inhibition of bacterial growth (ATP synthesis is not ensured). In contrast, the rates of O_2 consumption and DHA production are increased. More surprisingly, DHA synthesis is more accelerated than O_2 reduction. A succinate dehydrogenase activity, which has been already described by Williams and Rainbow (1964) in *G. oxydans*, was then observed. This succinate production establishes the necessity for the cell to possess a functional final acceptor system of reduced equivalents and demonstrates that succinate dehydrogenase and O_2 reductase may operate together as terminal oxidases. Under physiological conditions, only the O_2 reductase is induced and succinate production should be retroinhibited by the proton motive force in the presence of ATP.

The other problem dealt with by this paper is the physiological origin of DHA inhibition on the activities of *G. oxydans*. We have previously shown during *G. oxydans* culture that this compound firstly blocks microbial development and then inhibits glycerol oxidation (Claret et al. 1992). Thus, DHA inhibitions of growth and of substrate conversion must be studied separately.

It was shown that DHA inhibition of the ketone synthesis was mainly due to DHA inhibition of the membrane-bound enzyme responsible of glycerol oxidation: glycerol dehydrogenase. This inhibition may be due to the general phenomenon of retroinhibition by excess product or by a more direct interaction between the ketone and the membrane-bound enzyme. This phenomenon is probably linked to interaction between DHA and amine function localized on the enzymatic enzyme site. This result is not surprising considering the high reactivity of this compound with proteins, which confers on DHA its tanning properties (Meybeck 1977). In fact, the ketone spontaneously reacts with di-amino acids (arginine ...) to form Schiff's bases (Bobin et al. 1984; Blazejak and Sobcjak 1988) and so interacts with proteins by direct fixation.

Concerning the growth inhibition exerted by DHA, the lack of DHA inside the cell contradicts the hypothesis of an inhibition of the cytoplasmic enzymes involved in glycerol catabolism. The glycerol transport system, which has never been described before in the literature, was then investigated. Firstly, analysis of the changes in specific glycerol uptake rates with extracellular glycerol concentrations, determined in presence of the metabolic inhibitor KCN, establishes that the mode of glycerol penetration into the cells is a saturable process, and hence needs an enzymatic mediator, and is a facilitated diffusion process. KCN has the additional effect of inhibiting the proton gradient across the cell membrane. This inhibition does not prevent the transport of glycerol across the membrane. Consequently the mechanism by which this substrate penetrates bacteria is not an active transport but a facilitated diffusion phenomenon. Such a mode of glycerol transport has already been described for aerobic micro-organisms such as *Escherichia coli* (Sanno et al. 1968; Richey and Lin 1972), *Bacillus subtilis* (Saheb 1972) or *Nocardia asteroides* (Calmes and Deal 1972). Secondly, the enzymatic system involved in glycerol uptake is highly sensitive to DHA, which was shown by the 50% decrease in the specific glycerol uptake rate in the presence of DHA concentrations near to 1 mM. This very pronounced inhibition by DHA of the glycerol transport system is the main cause of bacterial growth inhibition and indicates that this phenomenon was the limiting step in the DHA production process using *G. oxydans*, as previously described (Claret et al. 1993). Nevertheless, bacterial growth is possible because DHA production during the first stage of the culture is low and because a residual glycerol transport phenomenon is observed when the DHA concentration in the culture medium is high.

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