Arch Microbiol (1987) 147:268–275

Archives of
Microbiology



# Overflow metabolism during anaerobic growth of *Klebsiella aerogenes* NCTC 418 on glycerol and dihydroxyacetone in chemostat culture

**H. Streekstra [1], M. J. Teixeira de Mattos [1], O. M. Neijssel [1], and D. W. Tempest [2]**

[1] Laboratorium voor Microbiologie, Universiteit van Amsterdam, Nieuwe Achtergracht 127, NL-1018 WS Amsterdam, The Netherlands
[2] Department of Microbiology, University of Sheffield, Sheffield, S10 2TN, UK

**Abstract.** *Klebsiella aerogenes* NCTC 418 was grown anaerobically in chemostat culture with glycerol as source of carbon and energy. Glycerol-limited cultures did not ferment the carbon source with maximal efficiency but produced considerable amounts of 1,3-propanediol. The fraction of glycerol converted to this product depended on the growth rate and on the limitation: faster growing cells produced relatively more of this compound. Under glycerol excess conditions the energetic efficiency of fermentation was decreased due to the high 1,3-propanediol excretion rate. Evidence is presented that 1,3-propanediol accumulation exerts a profound effect on the cells' metabolic behaviour.

When steady state glycerol-limited cultures were instantaneously relieved of the growth limitation a vastly enhanced glycerol uptake rate was observed, accompanied by a shift in the fermentation pattern towards 1,3-propanediol and acetate. This observation was consistent with the extremely high glycerol dehydrogenase activity that was measured in vitro. Some mechanisms that could be responsible for the energy dissipation during this response are discussed.

**Key words:** *Klebsiella aerogenes* – Glycerol – Dihydroxyacetone – Chemostat culture – Metabolic uncoupling

When growing anaerobically, many organisms are able to ferment the carbon source to a variety of end products by means of branched fermentation pathways. The different branches of the fermentation generally are not equivalent with respect to the generation of energy or reducing equivalents. For instance, *Klebsiella aerogenes* has the biochemical potential to ferment glucose to products as different as acetate, 2,3-butanediol, ethanol, succinate, D-lactate and formate (or $CO_2$ and $H_2$) (Gottschalk 1979). However, glucose-limited chemostat cultures ferment this carbon source solely to acetate and ethanol (plus formate). This fermentation pattern yields net 3 ATP per glucose, which is the maximal attainable energetic efficiency that is compatible with maintenance of a redox balance in this organism (Teixeira de Mattos and Tempest 1983). Under all glucose-sufficient conditions a branched fermentation, leading to the additional production of 2,3-butanediol, D-lactate and succinate, was observed with an associated decreased efficiency of ATP formation from glucose dissimilation. This loss in energetic efficiency was more than compensated for by an increased rate of carbon substrate catabolism. Moreover, the apparent $Y_{ATP}$ values (gram cells formed per mol of ATP generated net) of all cultures growing (at a fixed rate) in the presence of excess glucose were substantially lower than those of a glucose-limited culture. This can be partly explained by the increased energetic demands of biosynthesis when anabolic substrates such as ammonia are present in low concentrations only (Tempest and Neijssel 1984). However, it was concluded that under certain carbon-excess conditions ATP dissipation (energy-spilling) also must occur (Teixeira de Mattos and Tempest 1983).

This was even more apparent with glucose-limited cultures that were transiently relieved of the growth limitation. Here, the rate of glucose catabolism doubled whilst the growth rate remained constant, and now D-lactate also was formed. Subsequent studies (Teixeira de Mattos et al. 1984) revealed that this D-lactate arose from dihydroxyacetone phosphate (DHAP) via methylglyoxal with a concomitant uncoupling of glucose fermentation from net ATP formation. It should be pointed out that this mechanism of bypassing substrate-level phosphorylation can be functional anaerobically only when the uncoupling sequence as a whole is redox neutral. This is indeed the case for lactate formation from glucose.

When *K. aerogenes* is growing anaerobically on glycerol an additional branching point of fermentation exists: after glycerol has entered the cell by (facilitated) diffusion, it can be either oxidized to dihydroxyacetone (DHA), and subsequently phosphorylated to yield DHAP, or it can be dehydrated to 3-hydroxypropionaldehyde. The latter compound is then reduced to 1,3-propanediol (Lin 1976). From the scheme shown in Fig. 1 it can be calculated that when glycerol is the carbon source the most efficient fermentation would be to ethanol (or succinate), yielding 1 ATP/glycerol. This efficiency cannot be attained by growing cells, because the formation of biomass [with an approximate elemental composition of $C_4H_7O_2N$ (Herbert et al. 1971)] from glycerol generates reducing equivalents that can only be reoxidized via the formation of 1,3-propanediol, a process that does not generate ATP. The stoichiometry of net ATP formation must therefore be lower than 1 ATP per glycerol fermented.

From the above it will be clear that when glycerol and glucose are compared as carbon sources for the growth of *K. aerogenes*, they show a number of differences, in both uptake characteristics and catabolism. Moreover, during

*Offprint requests to*: H. Streekstra

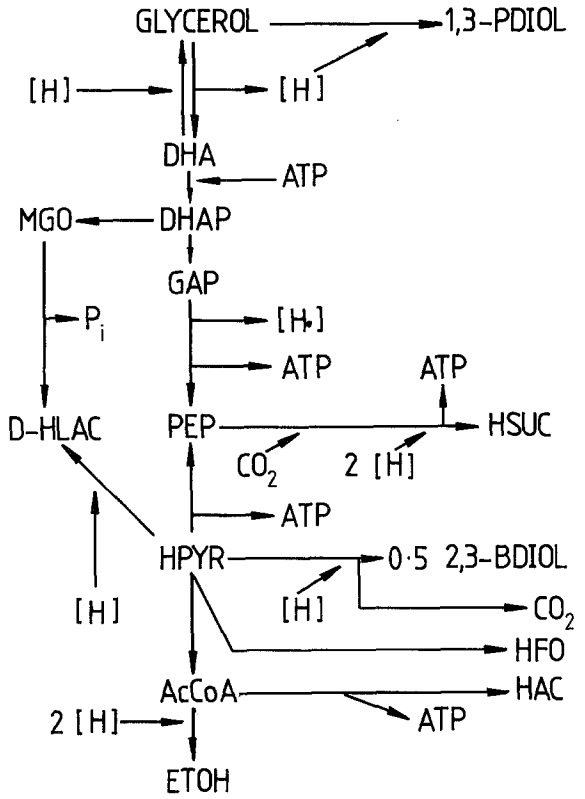


**Fig. 1.** Fermentation pathways of glycerol by *Klebsiella aerogenes*. *DHA* dihydroxyacetone, *DHAP* dihydroxyacetonephosphate, *1,3-PDIOL* 1,3-propanediol, *2,3-BDIOL* 2,3-butanediol, *GAP* glyceraldehyde-3-phosphate, *PEP* phospho-*enol*-pyruvate, *HSUC* succinic acid, *HPYR* pyruvic acid, *HLAC* lactic acid, *AcCoA* acetyl-coenzyme A, *HFOR* formic acid, *HAC* acetic acid, *ETOH* ethanol, *MGO* methylglyoxal, $P_i$ inorganic phosphate, [*H*] $NADH_2$

growth on glycerol anabolism may be affected by the need for gluconeogenesis.

In the present study we investigated the metabolic behaviour of *K. aerogenes* growing anaerobically in chemostat culture with glycerol as the sole source of carbon and energy under steady state and transient state conditions.

## Materials and methods

### *Organism*

*Klebsiella aerogenes* NCTC 418 was maintained by monthly subculture on tryptic meat-digest agar slopes.

### *Growth conditions*

Organisms were cultured in a 500 ml Porton-type chemostat (Herbert et al. 1965), in a 500 ml Modular Fermenter 500 Series II or 700 ml Series III (L. H. Engineering Co. Ltd., Stokes Poges, Bucks, England). Carbon-limited conditions were obtained by using simple salts media as specified by Evans et al. (1970), containing 15 g/l glycerol or 10 g/l DHA. For glycerol excess conditions 20 g/l glycerol was used unless specified otherwise. The concentration of the growth-limiting nutrient was usually half that prescribed by Evans et al. (1970). The pH value of the culture was maintained automatically at $6.8 \pm 0.1$ and the temperature was set at 36° C. The cultures were stirred at 1000 – 1400 rpm. Silicone antifoaming agent (BDH, Poole, England) was added to glycerol sufficient cultures to prevent excessive foaming. Anaerobiosis was maintained by the method described previously (Teixeira de Mattos and Tempest 1983).

### *Analyses*

Steady state bacterial dry weight was measured by the procedure of Herbert et al. (1971). Glycerol, dihydroxyacetone, D-lactate, acetate, succinate and ethanol were assayed enzymatically by the methods of Wieland (1974), Wieland and Witt (1974), Gawehn and Bergmeyer (1974), Holz and Bergmeyer (1974), Williamson (1974) and Bernt and Gutmann (1974), respectively. 2,3-Butanediol and 1,3-propanediol were assayed by gas chromatography according to Carlsson (1973). The above-mentioned compounds and formate were also determined by HPLC (LKB, Bromma, Sweden) with an Aminex HPX 87H Organic Acid Analysis Column (Biorad, Richmond, USA), using an 2142 Refractive Index Detector (LKB, Bromma, Sweden), an SP4270 Integrator (Spectra Physics, San Jose, USA) and 5 mM $H_2SO_4$ (Merck, Darmstadt, FRG) as eluent, at a temperature of 55° C. Carbon dioxide production by the cultures was determined by passing the effluent gas from the fermenter through an Infralyt T carbon dioxide analyzer (Junkalor, Dessau, GDR). Protein was assayed according to Gornall et al. (1949).

### *Pulse procedures*

In order to relieve instantaneously the growth limitation extant in a steady state glycerol-limited culture, 5 ml of a sterilized glycerol solution was injected by means of a syringe into the culture, providing an end concentration of about 40 mM. Simultaneously the medium pump was switched off. Every 2 min samples were withdrawn from the culture. The optical density was measured at 540 nm ($OD^{540}$), (Ultrospec 4051 Spectrophotometer, LKB, Bromma, Sweden), and cells were sedimented within 1 min using an Eppendorf 5412 bench centrifuge. Supernatants were decanted and refrigerated to be analyzed later. Growth rates were calculated from the $OD^{540}$ and checked by measuring bacterial dry weight before and after the pulse.

### *Enzyme activities*

To obtain cell-free extracts, cells were taken from a steady-state culture, centrifuged (5000 rpm, 10 min), washed with 50 mM phosphate buffer (pH = 7.0), sonified 6 times for 30 s at 75 W on a B 12 Sonifier (Branson, Danbury, USA) with intermittent 30 s periods of cooling in an ice-water mixture, and centrifuged at 20,000 rpm for 10 min. The supernatant was used for the determination of enzyme activities. Incubations of cell-free extracts with methylglyoxal (5 mM) were performed both in the presence and absence of 0.125 mM NADH. Otherwise the procedure of Cooper and Anderson (1970) was followed. Glycerol dehydrogenase activity was measured by determining the enzymic reduction of DHA (50 mM) in a 50 mM phosphate buffer (pH = 7.0) by 0.125 mM NADH on a Model 24 Double Beam Spectrophotometer (Beckman, Fullerton, USA). The enzymic reduction of acetol, methylglyoxal and pyruvate was measured by identical procedures. The enzymic oxidation of 50 mM glycerol and 1,2-propanediol in the presence

**Table 1.** Rates of glycerol utilization and of product formation expressed in anaerobic glycerol-limited chemostat cultures of *Klebsiella aerogenes* at various dilution rates

| $q$ | $D$ | | | | |
|---|---|---|---|---|---|
| | 0.10 | 0.18 | 0.30 | 0.44 | 0.62 |
| Glycerol | 18 | 31 | 49 | 60 | 101 |
| Acetate | 2.8 | 5.9 | 9.7 | 12 | 20 |
| Ethanol | 7.1 | 9.0 | 11 | 13 | 18 |
| 1,3-Pdiol | 6.6 | 13 | 23 | 25 | 50 |
| Succinate | 0.4 | 1.0 | 1.5 | 1.7 | 2.0 |
| $NADH_2/NAD$ | 1.05 | 1.08 | 1.00 | 1.20 | 0.96 |
| C-rec | 101 | 102 | 100 | 96 | 97 |
| Eff | 0.84 | 0.84 | 0.80 | 0.81 | 0.73 |
| $Y_{glycerol}$ | 5.4 | 6.0 | 6.2 | 7.4 | 6.1 |
| $Y_{ATP}$ | 6.8 | 7.6 | 8.4 | 10 | 9.1 |

Specific rates ($q$) are expressed in mmol·g cells$^{-1}$·h$^{-1}$. Yield values with respect to glycerol consumed ($Y_{glycerol}$) and ATP net synthesized ($Y_{ATP}$) are expressed in g cells·mol$^{-1}$. Carbon recovery (C-rec) is given as percentage of the glycerol consumed that is retrieved in carbon products. Stoichiometry of energy generation (Eff) is expressed in mol ATP synthesized per mol glycerol fermented. Abbreviations as in Fig. 1

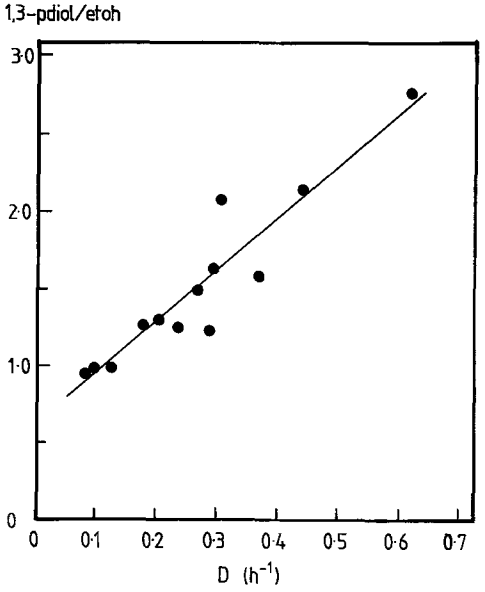


**Fig. 2.** The relationship between the 1,3-propanediol/ethanol production ratio and the growth rate of anaerobic, glycerol-limited cultures of *Klebsiella aerogenes*

of 0.125 mM NAD was measured in 0.4 M hydrazine, 0.2 M glycine (pH = 9.8).

## Results

*Klebsiella aerogenes* was grown anaerobically in chemostat cultures with glycerol as sole source of carbon and energy. Cultures were grown under glycerol limitation at various growth rates, or at a fixed growth rate that was limited by the availability of other nutrients. When steady state conditions had been attained, the amounts of fermentation products were quantitatively determined, and carbon- and redox-balances were constructed. The specific rate of glycerol consumption ($q_{glycerol}$) and the specific rates of formation of all products were calculated.

**Table 2.** Rates of glycerol utilization and of product formation expressed in variously limited anaerobic chemostat cultures of *Klebsiella aerogenes* growing at $D = 0.35$ h$^{-1}$

| $q$ | Limitation | | | |
|---|---|---|---|---|
| | Glycerol | Phosphate | Ammonia | Sulphate |
| Glycerol | 60 | 80 | 89 | 75 |
| Acetate | 11 | 18 | 20 | 16 |
| Ethanol | 11 | 2.4 | 12 | 12 |
| 1,3-Pdiol | 30 | 54 | 53 | 42 |
| Succinate | 1.2 | 0.8 | 0.9 | 0.8 |
| $NADH_2/NAD$ | 0.87 | 0.74 | 0.83 | 0.90 |
| C-rec | 97 | 100 | 100 | 100 |
| Eff | 0.72 | 0.57 | 0.66 | 0.69 |
| $Y_{glycerol}$ | 5.8 | 4.4 | 3.9 | 4.7 |
| $Y_{ATP}$ | 8.8 | 8.1 | 6.3 | 7.2 |

Abbreviations as in Fig. 1 and Table 1, parameters expressed as in Table 1

In contrast to previous findings with cultures of *K. aerogenes* growing anaerobically on glucose (Teixeira de Mattos and Tempest 1983), glycerol-limited cultures did not ferment glycerol with maximal efficiency (Table 1). For the observed yield of biomass, it can be calculated that the maximal stoichiometry of energy generation would be 0.92 ATP/glycerol fermented. Instead, only 0.74 to 0.84 ATP/glycerol was generated, due to the formation of 1,3-propanediol. Since the most efficient fermentation pathway would yield ethanol, the propanediol/ethanol ratio quantifies the loss of efficiency. It is noteworthy that this ratio increases with increasing growth rate (Fig. 2).

Glycerol-excess cultures excreted the same fermentation products as did glycerol-limited cultures, but the relative amounts of the various products were dependent on the nature of the growth limitation (Table 2). Whilst in phosphate-limited cultures almost the entire catabolic flux was directed to 1,3-propanediol and acetate, ammonia- and sulphate-limited cultures showed a fermentation pattern that was intermediate between phosphate and glycerol limitation. As with glucose-grown cells (Teixeira de Mattos and Tempest 1983) the $Y_{carbon\ source}$ (g cells synthesized/mol substrate fermented) and the $Y_{ATP}$ (g cells/mol ATP synthesized net) were invariably lower under carbon-excess conditions.

Under potassium limitation it was found that the fermentation pattern was similar to that found under phosphate limitation, and the $Y_{ATP}$ was the lowest observed (Fig. 3). However, increasing the glycerol input caused the specific uptake rate of glycerol to increase, even when as much as 40 mM glycerol was present in the culture fluid. Yet, the culture dry weight remained constant. This implies that a well-defined potassium limitation was not established.

In order to investigate whether this behaviour of potassium-limited cultures could be due to the high levels of 1,3-propanediol that were present in these cultures, 1,3-propanediol was added to the medium-supply of a glycerol

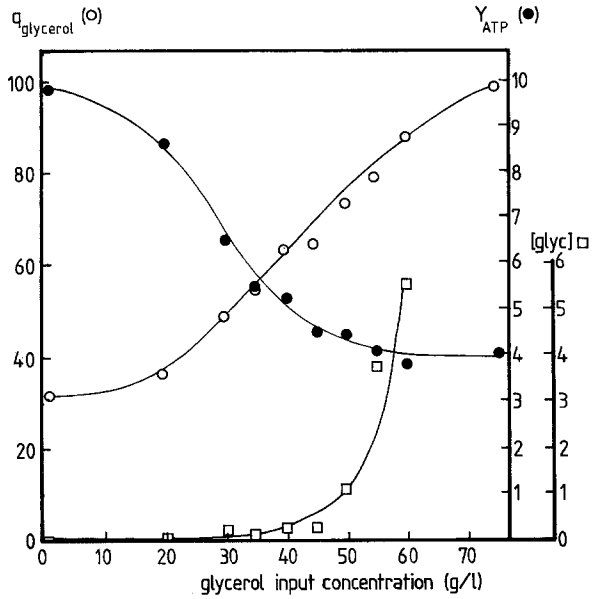


**Fig. 3.** The relationship between the glycerol input concentration (g/l) and $q_{glycoerol}$ (mmol · g cells$^{-1}$ · h$^{-1}$) (○), the extracellular glycerol concentration ([*glyc*], g/l) (□) and $Y_{ATP}$ (g/mol) (●), expressed in potassium-limited chemostat cultures of *Klebsiella aerogenes* growing at a dilution rate of 0.175 h$^{-1}$, with a potassium input concentration of 0.5 mM

**Table 3.** Rates of glycerol utilization and of product formation expressed in glycerol-limited anaerobic chemostat cultures of *Klebsiella aerogenes* growing at $D = 0.12$ h$^{-1}$ in the presence of various concentrations of 1,3-propanediol

| | Input 1,3-Pdiol | | |
|---|---|---|---|
| | 0 | 100 | 200 |
| 1,3-Pdiol$^{ext}$ | 65 | 155 | 238 |
| Glycerol$^{ext}$ | 0.25 | 1.2 | 3.9 |
| *q* | | | |
| Glycerol | 30 | 36 | 43 |
| Acetate | 5.4 | 5.1 | 2.5 |
| Ethanol | 11 | 15 | 29 |
| 1,3-Pdiol | 13 | 12 | 6.6 |
| Succinate | 0.9 | 1.6 | 1.6 |
| D-Lactate | 0 | 0.2 | 0.3 |
| C-rec | 103 | 99 | 97 |
| Eff | 0.76 | 0.81 | 0.87 |
| $Y_{glycerol}$ | 4.2 | 3.4 | 2.7 |
| $Y_{ATP}$ | 5.5 | 4.2 | 3.1 |

The concentration of 1,3-propanediol in the growth medium (Input 1,3-pdiol) and extracellular concentrations (1,3-pdiol$^{ext}$ and glycerol$^{ext}$) are expressed in mM. Abbreviations as in Fig. 1 and Table 1, parameters expressed as in Table 1

limited culture growing at a growth rate of 0.12 h$^{-1}$ (Table 3). This caused a substantial increase in the steady state extracellular glycerol concentration, while the culture dry weight decreased. As a result, the rate of ethanol formation increased, whereas the formation rates of 1,3-propanediol and acetate decreased.

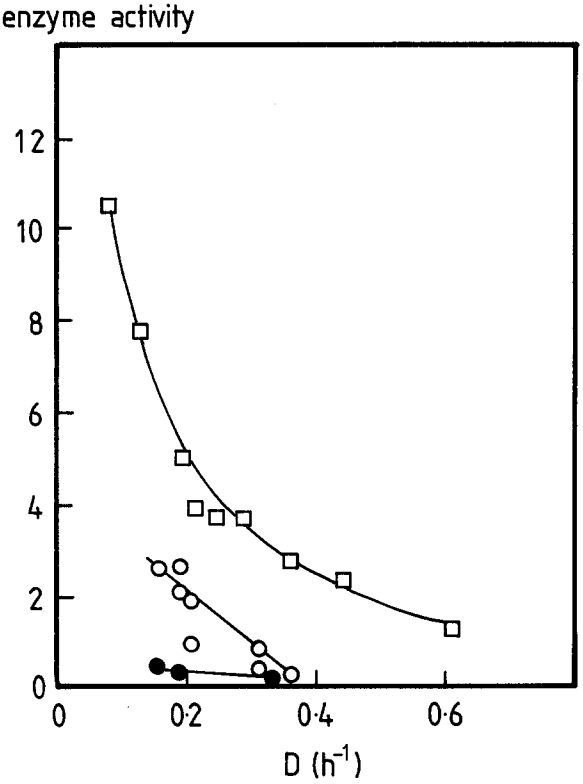


**Fig. 4.** The relationship between the growth rate and the activity of glycerol dehydrogenase (□) and methylglyoxal disimilation [(○) in the presence of $NADH_2$, (●) without $NADH_2$], respectively, in cell-free extracts of *Klebsiella aerogenes*, grown anaerobically under glycerol limitation. Enzyme activites are expressed as μmol · mg protein$^{-1}$ · min$^{-1}$

An important difference between carbon-limited growth on glycerol and glucose resides in the affinity of the primary metabolic enzymes: glucose is taken up by the phosphotransferase system, which has a high affinity for glucose, the apparent $K_m$ being 10 μM (Postma and Lengler 1985). Glycerol, however, enters the cell by (facilitated) diffusion and the primary metabolic enzyme, glycerol dehydrogenase, has an apparent $K_m$ of 2 to 45 mM, depending on the intracellular $NH_4^+$ concentration (Lin and Magasanik 1960). In glucose-limited chemostat cultures growing at low dilution rates, the extracellular glucose concentration is as low as 2 μM (Rutgers et al. 1987). In order to investigate the effect of the large differences in affinity of the primary metabolic enzymes for glucose and glycerol, the extracellular glycerol concentration in a glycerol-limited chemostat culture growing at a growth rate of 0.2 h$^{-1}$ was determined. This concentration was found to be only 0.3 mM. Significantly, the glycerol dehydrogenase activity in glycerol-limited cells was extremely high and increased as the growth rate (i.e. the external glycerol concentration) decreased (Fig. 4). Although the in vitro glycerol dehydrogenase activity was measured in the absence of $NH_4^+$ and $Mn^{2+}$, which are known to markedly activate this enzyme (Lin and Magasanik 1960; Hueting et al. 1978), the in vitro activity was about 25- ($D$ = 0.1 h$^{-1}$) to 1.4- ($D$ = 0.62 h$^{-1}$) fold higher than the activity actually expressed in vivo. For comparison, in ammonia-limited cells growing at 0.2 h$^{-1}$ the glycerol dehydrogenase activity was only half that found under glycerol-limitation for $D = 0.62$ h$^{-1}$. No glycerol

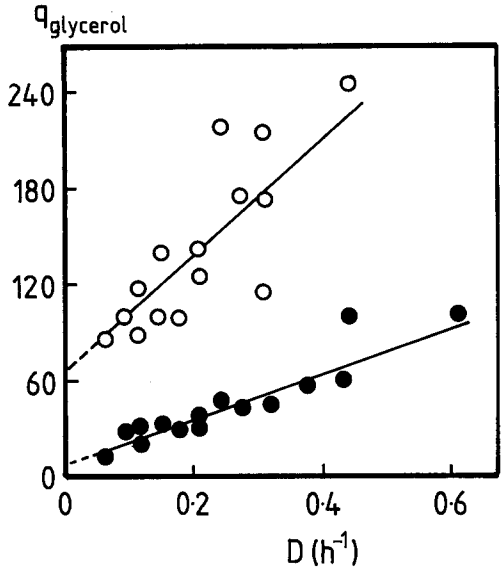


**Fig. 5.** The relationship between the growth rate and the specific rate of glycerol consumption ($q$) of glycerol-limited anaerobic cultures of *Klebsiella aerogenes*. (●) Specific uptake rate at steady state, (○) specific uptake rate after addition of 40 mM glycerol to the culture

kinase activity, the primary catabolic enzyme during aerobic glycerol-limited growth, was observed, in accordance with the findings of Neijssel et al. (1975).

When glucose-limited anaerobic cultures of *K. aerogenes* were relieved of their growth limitation, by a sudden addition of a cell-saturating amount of glucose, the uptake rate of glucose increased instantaneously, and substantially, whilst, at growth rates below 0.5 $h^{-1}$, the rate of cell synthesis remained constant. This increased rate of substrate catabolism was not accompanied by an increase in the rate of ATP formation due to the functioning of the methylglyoxal bypass, leading to D-lactate excretion (Teixeira de Mattos et al. 1984). Similar results were obtained with anaerobic glycerol-limited cultures to which a saturating amount of glycerol was added: again, *K. aerogenes* possessed the potential to consume carbon substrate (glycerol) at a vastly enhanced rate, without an increase in growth rate (Fig. 5). Indeed, often the growth rate was lower than in steady state and in a number of experiments growth stopped completely. In the latter case, resumption of the steady state medium flow caused the culture to wash out. After relief of the glycerol limitation, virtually all (typically 90%) glycerol could be retrieved as 1,3-propanediol and acetate, and ethanol formation stopped completely.

Since the vast increase in glycerol uptake rate is not accompanied by an increase in growth rate, a question arises as to how energy is dissipated. Therefore we investigated whether the methylglyoxal bypass could play a role here. The enzymes of the methylglyoxal bypass, methylglyoxal synthase (data not shown) and glyoxalase (Fig. 4), proved to be present in glycerol-limited cell free extracts at levels comparable to those in glucose-limited cells. However, although often D-lactate formation was observed following a glycerol pulse, the formation rate was small compared to the high glycerol uptake rate. In view of the fact that 10% of the glycerol could not be accounted for, it may be that some unknown product was formed following a glycerol pulse. Particularly, since lactate formation from glycerol is not a redox neutral process, the possibility of methylglyoxal reduction was investigated. It was found that cell-free extracts from glycerol-limited cells were able to reduce methylglyoxal at the expense of NADH at a high rate, and that this activity correlated with glycerol dehydrogenase activity (Fig. 4), and not with the activities of other dehydrogenases, such as pyruvate reductase (soluble NAD-dependent D-lactate dehydrogenase). Furthermore, it could be shown that both cell free extracts from *K. aerogenes* and purified glycerol dehydrogenase from *Enterobacter aerogenes* (Boehringer, Mannheim, FRG) were able to oxidize glycerol and 1,2-propanediol at the expense of NAD and to reduce DHA, methylglyoxal and acetol at the expense of NADH. Of these reactions the oxidation of glycerol and 1,2-propanediol and the reduction of DHA were stimulated by $Mn^{2+}$ in both enzyme preparations, whereas the reduction of methylglyoxal and acetol were not (data not shown).

To elucidate the effect of the specific properties of glycerol dehydrogenase on the metabolism of glycerol-grown organisms, *K. aerogenes* was grown on DHA, the product of the glycerol dehydrogenase reaction. It was found that DHA-limited cultures excreted substantial amount of 1,3-propanediol, obviously with glycerol as intermediate (Table 4). It should be borne in mind that in this case formation of 1,3-propanediol does not influence the stoichiometry of ATP generation, because its synthesis now consumes two reducing equivalents and thereby allows a compensating increase in acetate production. As a result, the maximal efficiency (1.5 ATP/DHA fermented) was reached.

**Table 4.** Rates of DHA utilization and of product formation expressed in DHA-limited anaerobic chemostat cultures of *Klebsiella aerogenes* growing at $D = 0.34\ h^{-1}$

| $q$ | DHA limitation |
|---|---|
| DHA | 24 |
| Acetate | 13 |
| Ethanol | 2.9 |
| 1,3-Pdiol | 4.6 |
| Succinate | 1.0 |
| Glycerol | 0.2 |
| $NADH_2$/NAD | 1.08 |
| C-rec | 108 |
| Eff | 1.56 |
| $Y_{DHA}$ | 14 |
| $Y_{ATP}$ | 9.9 |

Abbreviations as in Fig. 1 and Table 1, parameters expressed as in Table 1

## Discussion

Under aerobic growth conditions, *Klebsiella aerogenes* will react to different limitations by excreting more or less specific partially oxidized end products, so called overflow metabolites (Neijssel and Tempest 1975). Overflow metabolism can be seen as a mechanism for uncoupling catabolism from anabolism under conditions in which the energy source is present in excess of the growth requirement. Anaero-

bically, this type of metabolic flexibility is constrained by the need for balancing the flow of reductant. However, the occurrence of branched fermentation pathways can be interpreted as a similar mechanism. If the biochemical make-up of the organism is sufficiently known, it is possible to predict the energetically most efficient pattern for any given energy source. Overflow metabolism may then be defined as the departure from this pattern (Teixeira de Mattos et al. 1987).

For instance, 2,3-butanediol partly replaces acetate when glucose is supplied in excess of the growth requirement, and D-lactate is excreted in small amounts by glucose-limited cultures at high growth rates (Teixeira de Mattos and Tempest 1983). A similar fermentation shift can be observed with the so-called homolactic streptococci, which produce acetate and ethanol at low growth rates and lactate at high growth rates (Thomas et al. 1979).

For anaerobic growth on glycerol to proceed, 1,3-propanediol formation is required to oxidize the reducing equivalents set free during biosynthesis. It appears, however, that the amount of 1,3-propanediol that is synthesized is highly variable, and dependent on the growth rate and the nature of the limitation. The conversion of glycerol to 1,3-propanediol is maximal under phosphate limitation and in environments with a low availability of potassium. This regulatory pattern is highly reminiscent of that of glucose dehydrogenase in aerobically grown *K. aerogenes* (Hommes et al. 1985). This pattern has been interpreted as glucose dehydrogenase acting as a low impedance energy generating system, operating under conditions in which a high rate of energy generation is required. We suggest that 1,3-propanediol excretion serves a similar role: its synthesis does not generate ATP, but it lowers the impedance by increasing the rate of NADH oxidation, thereby allowing a higher rate of DHA, and ultimately acetate, synthesis at the expense of a lowered efficiency of ATP generation. Moreover, the observation that the relative amount of glycerol that is fermented to 1,3-propanediol increases with increasing growth rate, again indicates a role in sustaining high rates of energy generation. For these reasons 1,3-propanediol synthesis can be termed overflow metabolism in the sense defined above.

The fraction of the glycerol that is fermented to 1,3-propanediol is minimal under glycerol limitation at low growth rates. However, even under these conditions, more 1,3-propanediol is excreted than is necessary to regenerate NAD for biosynthesis. Thus, as opposed to the behaviour under glucose limitation, glycerol-limited cultures show overflow metabolism as well. It could be that this behaviour is a reflection of the specific kinetics of the enzymes acting directly on glycerol: whereas the glycerol dehydrogenase has an apparent $K_m$ for glycerol of more than 10 mM, the glycerol dehydratase has a $K_m$ of 40 – 100 μM (Korsova et al. 1982). Thus, it can be argued that the combined action of glycerol dehydratase and 1,3-propanediol dehydrogenase serves to increase the overall affinity for glycerol uptake. This is very well illustrated by the fact that the presence of elevated levels of 1,3-propanediol in glycerol-limited cultures causes the extracellular glycerol concentration to increase. The concomitant decrease of the $Y_{ATP}$ may be explained by an additional energetic stress requiring a higher energy generation rate. At present, we cannot discriminate between a toxic effect of this product and an energy investment needed for its excretion. In either case it should be emphasized that the extracellular 1,3-propanediol concentration partly determines the metabolic behaviour which is therefore not completely dependent on the nature of the experimentally imposed growth conditions. A similar reasoning holds for the metabolic behaviour of putatively potassium-limited cultures.

During anaerobic growth on glycerol, glycerol dehydrogenase, DHA kinase, glycerol dehydratase and 1,3-propanediol dehydrogenase are induced (Lin 1976). It has been reported that DHA is the actual inducer of these four enzymes, therefore called the dha-system (Forage and Foster 1982). Our observation that DHA-limited cultures excrete 1,3-propanediol is in accordance with this regulation. However, we also observed that the glycerol dehydrogenase is induced to extremely high levels under glycerol limitation at low growth rates. Whereas this metabolic reaction can be rationalized in terms of the selective pressure to optimize substrate scavenging capacity on the one hand and the extraordinarily low affinity of the glycerol dehydrogenase for glycerol on the other, it suggests that the mechanism of induction is more complex than previously appreciated, and that perhaps some form of derepression is involved.

Moreover, it has been suggested that the dha-system serves to dismutate two molecules of glycerol to one molecule of 1,3-propanediol and one molecule of DHA in an overall redox neutral process, after which fermentation of DHA to ethanol and acetate can proceed (Forage and Foster 1982), leading to a 1,3-propanediol/ethanol ratio of 2. That this is too rigid a model for the role of 1,3-propanediol excretion is suggested by the observation that *Lactobacillus* species form 1,3-propanediol from glycerol, but cannot use glycerol for growth (Schutz and Radler 1984). Furthermore, our results indicate that for *K. aerogenes* this ratio only is observed with glycerol-limited cultures growing at high growth rates: at lower growth rates glycerol-limited cultures produce less 1,3-propanediol, whilst glycerol-excess cultures produce more. Finally, this model obviously does not hold true for the conversion of DHA to 1,3-propanediol under DHA-limited growth conditions.

The $Y_{ATP}$ values of glycerol-grown cells are low as compared with cells grown on glucose (Teixeira de Mattos and Tempest 1983). Only under carbon limitation at low growth rates are they similar. The $Y_{ATP}$ value reflects the energetic demands of both biosynthesis and the environment. Biosynthesis from glycerol requires gluconeogenesis, while biosynthesis from glucose does not. The need for gluconeogenesis can explain the observed differences in the values of $Y_{ATP}$ only if faster growing cells contain relatively more of a polymer for which gluconeogenesis is required. Indeed, with aerobic glycerol-limited cultures of *K. aerogenes* it was found that at higher growth rates cells contained more RNA whereas the amounts of DNA and protein remained almost constant (Herbert 1961). However, since over the range of growth rates investigated the RNA content increases only twofold, it cannot account completely for the observed discrepancy.

Our results suggest that the presence of 1,3-propanediol exerts a negative effect on the value of $Y_{ATP}$. First, addition of this compound to a glycerol-limited culture caused a lowering of the $Y_{ATP}$. Second, the increase in the 1,3-propanediol/ethanol ratio with increasing growth rate correlated with an increase in the discrepancy between the $Y_{ATP}$ values expressed in glycerol- and glucose-limited cultures. Third, glycerol-excess cultures expressed lower $Y_{ATP}$ values

than cultures growing on glucose with the same growth limitation, and under the former conditions the rate of formation of 1,3-propanediol was high. Finally, it was the major fermentation product after relief of glycerol limitation, when some form of ATP dissipation must take place. This suggestion is compatible with the observation that the diol dehydratase of *K. pneumoniae*, which is very similar to glycerol dehydratase [though not identical (Toraya and Fukui 1977)] is membrane-associated on the cytosolic side (McGee and Richards 1981), as is the diol dehydratase from *Clostridium glycolicum* (Hartmanis and Stadtman 1986). In addition to the above-mentioned arguments, it is noteworthy that DHA-limited cells, for which the requirement for gluconeogenesis can be assumed to be similar to that under glycerol limitation, show $Y_{ATP}$ values intermediate between glycerol- and glucose-limited cells. Under these conditions, 1,3-propanediol is excreted at much lower rates.

With glycerol-limited cells that are relieved of their limitation, an alternative possibility for dissipating energy would be the methylglyoxal bypass. Although in these cells the activity in vitro of this sequence is comparable to that under glucose limitation, lactate formation was not always observed. However, it should be realized that a low activity in vivo of the methylglyoxal bypass would be sufficient to dissipate energy along with 1,3-propanediol formation. Moreover, we have shown that glycerol dehydrogenase is able to reduce methylglyoxal at the expense of NADH, which, if the mechanism is similar to the reduction of DHA, would yield lactaldehyde as a product, in an overall redox-neutral process. This latter compound may not be detectable with the methods used in this work. Whatever its nature, it is clear that an effective mechanism of energy dissipation must exist in view of the fact that the glycerol uptake rate increased vastly, and reached extremely high absolute levels, while the growth rate remained constant or even decreased.

## References


Bernt E, Gutmann I (1974) Äthanol, Bestimmung mit Alkohol-Dehydrogenase und NAD. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, 3rd edn, vol 2. Verlag Chemie, Weinheim, pp 1545–1548

Carlsson J (1973) Simplified gas chromatographic procedure for identification of bacterial metabolic products. J Appl Microbiol 25:287–289

Cooper RA, Anderson A (1970) The formation and catabolism of methylglyoxal during glycolysis in *Escherichia coli*. FEBS Lett 11:273–276

Evans CGT, Herbert D, Tempest DW (1970) The continuous culture of microorganisms. 2. Construction of a chemostat. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 2. Academic Press, London New York, pp 277–327

Forage RG, Foster MA (1982) Glycerol fermentation in *Klebsiella pneumoniae*: Functions of the coenzyme $B_{12}$-dependent glycerol and diol dehydratase. J Bacteriol 149:413–419

Gawehn K, Bergmeyer HU (1974) D-(−)-Lactat. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, 3rd edn, vol 2. Verlag Chemie, Weinheim, pp 1538–1541

Gornall AG, Bardawill CJ, David MA (1949) Determination of serum proteins by means of the biuret reaction. J Biol Chem 177:751–766

Gottschalk G (1979) Bacterial metabolism. Springer, Berlin Heidelberg New York

Hartmanis MGN, Stadtman TC (1986) Diol metabolism and diol dehydratase in *Clostridium glycolicum*. Arch Biochem Biophys 245:144–152

Herbert D (1961) The chemical composition of micro-organisms as a function of their environment. In: Meynell GG, Gooder H (eds) Microbial reaction to the environment. 11th Symp Soc Gen Microbiol. Cambridge University Press, Cambridge, pp 391–416

Herbert D, Phipps PJ, Tempest DW (1965) The chemostat: design and instrumentation. Lab Pract 14:1150–1161

Herbert D, Phipps PJ, Strange RE (1971) Chemical analysis of microbial cells. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 5b. Academic Press, London New York, pp 209–344

Holz G, Bergmeyer HU (1974) Acetat. Bestimmung mit Acetat-Kinase und Hydroxylamin. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, 3rd edn, vol 2. Verlag Chemie, Weinheim, pp 1574–1578

Hommes RWJ, van Hell B, Postma PW, Neijssel OM, Tempest DW (1985) The functional significance of glucose dehydrogenase in *Klebsiella aerogenes*. Arch Microbiol 143:163–168

Hueting S, de Lange T, Tempest DW (1978) Properties and regulation of synthesis of the glycerol dehydrogenase present in *Klebsiella aerogenes* NCTC 418, growing in chemostat culture. FEMS Microbiol Lett 4:185–189

Korsova TL, Vorontsov EA, Gurevitch VM, Poznanskaya AA (1982) Substrate specificity of adenosylcobalamin dependent glycerol dehydratase interaction with enantiomers of 1,2-propanediol. Biokhimiya 47:379–384

Lin ECC (1976) Glycerol dissimilation and its regulation in bacteria. Ann Rev Microbiol 30:535–578

Lin ECC, Magasanik B (1960) The activation of glycerol dehydrogenase of *Aerobacter aerogenes* by monovalent ions. J Biol Chem 235:1820–1823

McGee DE, Richards JH (1981) Purification and subunit characterization of propanediol dehydratase, a membrane-associated enzyme. Biochemistry 20:4293–4298

Neijssel OM, Tempest DW (1975) The regulation of carbohydrate metabolism in *Klebsiella aerogenes* NCTC 418 organisms, growing in chemostat culture. Arch Microbiol 106:251–258

Postma PW, Lengeler JW (1985) Phosphoenolpyruvate: carbohydrate phosphotransferase system of bacteria. Microbiol Rev 49:232–269

Rutgers M, Teixeira de Mattos MJ, Postma PW, van Dam K (1987) Establishment of the steady state in glucose-limited chemostat cultures of *Klebsiella aerogenes*. J Gen Microbiol (in press)

Schutz H, Radler F (1984) Anaerobic reduction of glycerol to propanediol-1,3 by *Lactobacillus brevis* and *Lactobacillus buchneri*. System Appl Microbiol 5:169–178

Teixeira de Mattos MJ, Tempest DW (1983) Metabolic and energetic aspects of the growth of *Klebsiella aerogenes* NCTC 418 on glucose in anaerobic chemostat cultures. Arch Microbiol 134:80–85

Teixeira de Mattos MJ, Streekstra H, Tempest DW (1984) Metabolic uncoupling of substrate level phosphorylation in anaerobic glucose-limited chemostat cultures of *Klebsiella aerogenes* NCTC 418. Arch Microbiol 139:260–264

Teixeira de Mattos MJ, Streekstra H, Neijssel OM, Tempest DW (1987) The link between the efficiency of ATP generation and metabolic flexibility during anaerobic growth on various carbon sources. In: Stowell JD, Beardsmore AJ, Keevil CW, Woodward JR (eds) Carbon substrates in biotechnology. Soc Gen Microbiol, vol 21. IRL Press, Oxford

Tempest DW, Neijssel OM (1984) The status of $Y_{ATP}$ and maintenance energy as biologically interpretable phenomena. Ann Rev Microbiol 38:459–486

Thomas TD, Ellwood DC, Longyear VMC (1979) Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat culture. J Bacteriol 138:109–117

Toraya T, Fukui S (1977) Immunochemical evidence for the difference between coenzyme $B_{12}$-dependent diol dehydratase and glycerol dehydratase. Eur J Biochem 76:285–289

Wieland O (1974) Glycerol: UV Methode. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, 3rd edn, vol 2. Verlag Chemie, Weinheim, pp 1448–1450

Wieland O, Witt I (1974) Dihydroxyaceton. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, 3rd edn, vol 2. Verlag Chemie, Weinheim, pp 1487–1489

Williamson JR (1974) Succinat. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, 3rd edn, vol 2. Verlag Chemie, Weinheim, pp 1661–1666

Received September 22, 1986/Accepted January 20, 1987