Effect of Starvation on the Viability and Cellular Constituents of *Zymomonas anaerobia* and *Zymomonas mobilis*

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SUMMARY

Zymomonas anaerobia and *Z. mobilis*, grown on glucose + peptone + yeast-extract medium, degraded no endocellular carbohydrate, DNA and protein even in prolonged starvation. No significant qualitative changes in protein content during starvation were detected by disc gel electrophoresis of crude extracts. Both organisms had a high content of RNA (22 % w/w) which was degraded on starvation. In *Z. anaerobia* RNA decreased linearly to 5 % of the dry weight in 125 hr. With *Z. mobilis*, half the RNA was degraded in the first 24 hr of starvation after which time the decline was much slower. $MgCl_2$ (33 mM) prevented RNA breakdown. During growth, the intracellular ATP concentration increased from 0.5 to 1.0 $\mu g./mg.$ dry wt, but began to decrease exponentially in the last generation before growth ceased because of glucose exhaustion. Intracellular ATP content correlated with viability determined by slide culture. The addition of 33 mM- $MgCl_2$ to the starvation medium did not affect ATP content, but increased viability. On prolonged starvation (up to 7 days), populations whose viability had fallen to 3 % possessed unimpaired ability to produce ATP from glucose; only after even longer starvation periods was this ability impaired.

INTRODUCTION

During recent years considerable attention has been focused on the nature of the endogenous metabolism of micro-organisms (for reviews, see Dawes & Ribbons, 1962, 1964) although this work has been confined mainly to aerobic and facultatively anaerobic organisms. By investigating the changes in chemical composition which occur during starvation an insight can be afforded into the substrates utilized in endogenous metabolism and their relation to the viability of the organisms. Anaerobic bacteria, which obtain their energy by substrate-level phosphorylation reactions which are well charted, ought to be particularly amenable to investigation, especially in relation to the problem of 'maintenance energy' (Dawes & Ribbons, 1964). Forrest & Walker (1965) studied the endogenous metabolism of the homofermentative organism *Streptococcus faecalis* which degrades glucose to lactate via the Embden-Meyerhof pathway, and Thomas (1968) has investigated *Streptococcus lactis*. Accordingly, for the present work, *Zymomonas anaerobia* (McGill, Dawes & Ribbons, 1965; McGill, 1966) and *Z. mobilis* (Stern, Wang & Gilmour, 1960; Dawes, Ribbons & Large, 1966) were chosen; they ferment glucose to ethanol and carbon dioxide by the Entner-Doudoroff (1952) pathway. Characteristic of this pathway is an energy yield of 1 mole ATP/mole glucose fermented, i.e. half that obtained by the Embden-Meyerhof pathway (Elsden & Peel, 1958).

Most of our work was done with *Z. anaerobia*, but where the results with *Z. mobilis* differed, they have been reported. A preliminary account of the earlier part of this work has appeared (Large & Dawes, 1966).

METHODS

Maintenance and growth of organisms. *Zymomonas anaerobia* (NCIB8227) was maintained as a stab culture in agar and also in liquid medium. The basal liquid medium contained (g./l.): D-glucose, 20; Difco Bactopeptone, 10; Difco yeast extract, 10. When required, it was solidified with 15 g. New Zealand agar substitute/l. (in the case of the slide cultures 10 g. Oxoid Agar No. 1/l. was used). Cultures were incubated at 30°. *Zymomonas mobilis* (NCIB 8938) was maintained in a similar manner. This organism grew with a doubling time of about 2 hr, compared with 3.5 hr for *Z. anaerobia*.

Growth of larger quantities of either organism was achieved by transfer of an inoculum from the stock culture to 20 ml. sterile liquid medium. When this culture was gassing vigorously (after about 12 hr) it was used to inoculate one or more litres of medium in round flat-bottomed flasks or aspirators filled to the neck. *Zymomonas anaerobia* attained stationary phase in 20 hr at 30°, while *Z. mobilis* took 12 to 15 hr.

For starvation experiments organisms were harvested aseptically in closed, sterile centrifuge bottles and the cultures were examined for contamination by phase-contrast microscopic examination of a wet smear.

Measurement of bacterial concentration. This was performed turbidimetrically with a Unicam SP 600 spectrophotometer at 570 nm. Extinctions were related to population density by a dry wt calibration curve.

Preparation of washed suspensions for starvation experiments. After the appropriate period of growth organisms were harvested aseptically and washed in sterile phosphate buffer (below), pH 6.8, and suspended in a suitable volume of the same buffer in a sterile measuring cylinder. In earlier experiments the suspension was then distributed into sterile 50 ml. conical flasks, which were flushed with N₂, sealed with sterile silicone rubber stoppers and shaken at 30°. In later experiments the apparatus described by Dawes & Holms (1958) was used in which nitrogen was bubbled through the suspension at 30° via a sintered glass aerator. This apparatus bore a tap at the base through which samples could be removed aseptically, and had a small side arm into which substrate could be introduced aseptically at the start of the experiment, and subsequently tipped into the suspension without admitting air to the flask.

Preparation of extracts and disc electrophoresis. *Zymomonas anaerobia* organisms (about 70 mg. dry wt) were suspended in 2.5 ml. ice-cold 67 mM-phosphate buffer (pH 6.8), disrupted for 1.5 min. in an M.S.E. 60 W ultrasonic disintegrator and centrifuged for 10 min. at 25,000g to remove whole cells and debris. Samples of the supernatant fluid containing 100 µg. protein were used for polyacrylamide disc electrophoresis as described by Davis (1964).

Determination of ATP. The ATP content of organisms was measured by the firefly luciferase technique based on the method of Forrest & Walker (1965). Cole, Wimpenny & Hughes (1967) pointed out that for accurate assay of intracellular ATP there should be as little lag as possible between harvesting of the organisms and extraction of the ATP; that, ideally, samples ought not to be harvested by centrifugation, and the samples should not be chilled. In the present work the method developed by Cole *et al.* (1967), in which growing bacteria were pipetted into HClO₄, did not extract

the ATP from *Z. anaerobia* satisfactorily, so they were centrifuged before extracting the ATP. This was done within 5 min., the organisms being centrifuged for 2 min. at 20,000g at room temperature in a Servall Superspeed SS-1 centrifuge. ATP was extracted from the unwashed packed organisms by using 0.6 N-H₂SO₄ as described by Forrest & Walker (1965) except that 20 mM-Na₃PO₄ was used to neutralize the acid instead of KOH.

The apparatus used was a high-gain photomultiplier tube (E.M.I. type 6097S) with a Nuclear Enterprises type N.E. 5353 high voltage supply (Damoglou & Dawes, 1968). The current output of the tube was measured after conversion to voltage on a Yellow Springs Model 80 laboratory recorder (Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio, U.S.A.). The firefly lantern extract and the estimation method were as described by Forrest & Walker (1965).

Viability determination by anaerobic slide culture. The slide culture method of Postgate, Crumpton & Hunter (1961) was modified slightly for anaerobic bacteria. Since *Zymomonas anaerobia*, long thought to grow only anaerobically (Shimwell, 1937, 1950) was found during this work to tolerate oxygen and to grow slowly under aerobic conditions, rigid precautions to ensure anaerobiosis during harvesting and preparation of the slide culture were not necessary. Starved suspensions were diluted 1/10 with sterile 67 mM-phosphate buffer (pH 6.8) and one drop applied to the agar on the slide. After drying and removal of excess moisture, the cultures were sealed with sterile buffer under circular coverslips, placed in Petri dishes and incubated in an anaerobic jar filled with oxygen-free N₂ at 22°, high viability samples for 48 hr, samples of low viability for 72 hr, and the numbers of divided and undivided bacteria (single or paired) were counted under a Watson Microsystem 70 phase-contrast microscope and the % viability calculated. No further increase in the % viability was noted when the incubation times were prolonged further.

Extraction of amino acid pool. Bacteria (5 mg. dry wt) were centrifuged down and suspended in 5 ml. water. After adding one drop of 2 N-H₂SO₄, the suspension was heated in a water bath at 100° for 10 min. with a glass bulb condenser on the tube. The cell debris was centrifuged down, and the supernatant fluid analysed for amino acids.

Chemical determinations. Ethanol was determined enzymically (Kaplan & Ciotti, 1957) by using liver alcohol dehydrogenase 340-L2 (Sigma-London Chemical Co.). Standards prepared from ethanol standardized by specific gravity determination were estimated simultaneously. Glucose in suspensions of starved bacteria was determined by the method of Nelson (1944), but for bacterial growth media the glucose oxidase + horseradish peroxidase method (Huggett & Nixon, 1957) with the Boehringer blood sugar test combination TC-M-II was used, since constituents of the peptone produced false positive reactions with the Nelson reagent. Pyruvate was estimated by the direct method of Friedemann & Haugen (1943), amino acids by the method of Yemm & Cocking (1955) and carbohydrate by the anthrone method of Trevelyan & Harrison (1952) as modified by Binnie, Dawes & Holms (1960). Ammonia was determined by the indophenol method of Chaney & Marbach (1962), polyphosphate as described by Harold (1960) and poly-β-hydroxybutyrate by the method of Law & Slepecky (1961), using the isolation procedure of Williamson & Wilkinson (1958). Cell protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) after suspensions had been boiled for 6 min. in 2.5 N-NaOH.

Bovine serum albumin was used as standard. Cold-acid-soluble nucleotides were estimated by extraction of 5 mg. dry wt bacteria in ice-cold 0.7 N-HClO₄ for 10 min. at 0°. After centrifugation at 25,000g, the residue was washed three times with 0.7 N-HClO₄, the supernatant fluids and washings combined and the extinction read at 260 nm. The residue was hydrolysed with 0.3 N-KOH for 90 min. at 37°. The RNA content was determined on the basis of the extinction at 260 nm., as described by Fleck & Munro (1962), with yeast RNA hydrolysed by the same procedure as a standard. DNA was estimated on the cold-HClO₄-washed residue by the indole method of Ceriotti (1952).

Materials and chemicals. Analytical reagent grade chemicals were used where available. Firefly lanterns and ATP were obtained from Sigma-London Chemical Co., (London, S.W.6) and NAD from the Boehringer Corporation (London) Ltd. (London, W.5).

Buffer. KH₂PO₄ (0.067 M) was adjusted to pH 6.8 with 5 N-NaOH.

RESULTS

Changes in cellular constituents during growth and starvation

Carbohydrate content. *Zymomonas anaerobia* contained about 4 % of its dry wt as carbohydrate, which did not change on starvation up to 118 hr in N₂. *Z. mobilis* contained about 5 % (w/w) carbohydrate, which decreased to about 3 % on starvation up to 118 hr under N₂.

Poly-β-hydroxybutyrate and polyphosphate. These polymers were absent from both organisms.

DNA content. *Zymomonas anaerobia* contained about 2.7 % (w/w) DNA, and this remained stable on starvation to at least 138 hr.

Protein content. The protein content of *Zymomonas anaerobia* was examined at various stages of growth and during starvation of the harvested and washed bacteria under N₂. During growth the protein content remained essentially constant at 65 to 69 % of the dry wt but decreased to 54 % late in the stationary phase. During starvation of washed suspensions the protein content did not change significantly during starvation up to 150 hr; this result was not altered when the cells were either shaken or left stationary. The protein content was not affected when the glucose concentration in the growth medium was varied from 1 to 5 % (w/v).

The effect of starvation on the qualitative pattern of individual proteins in the soluble fraction of cell extracts was examined by disc electrophoresis on polyacrylamide gel. In extracts of unstarved organisms about 14 different protein bands were distinguished, of which five were especially prominent. After 159 hr starvation, 13 bands were still evident and the same five bands were still prominent. There did not seem to be any major or significant changes in individual protein bands in samples taken during this period.

Amino acids and ammonia. The concentration of ammonia and amino acids in the hot-water-extractable pool of *Zymomonas anaerobia* did not change in starvation periods up to 24 hr. Variable amounts of ammonia (from 0.1 to 0.5 μmole/mg. dry wt) and amino acids (0.02 to 0.2 μmole/mg. dry wt) were released into the medium during these periods; in some experiments as much as 1 μmole ammonia/mg. dry weight was noted.

RNA and cold-acid-soluble nucleotide pool. *Zymomonas anaerobia* has a high content of RNA (17 to 25 %, average 22 %, of the dry wt of freshly harvested late exponential phase organisms). On prolonged starvation, the RNA content decreased drastically to as low as 3 % after 138 hr (Fig. 1A). During this period considerable amounts of material having an absorption maximum at 260 nm. were released into the medium, and when expressed as RNA this accounted for all the RNA degraded, since the cold-acid-soluble nucleotide pool remained essentially constant. The degradation of RNA began immediately and continued in a linear fashion during starvation. The degradation was suppressed when the organisms were starved in the presence of 33 mM-MgCl₂ (Fig. 1A).

Fig. 1. Effect of Mg²⁺ ions on the degradation of RNA by anaerobically starved *Z. anaerobia* (A) and *Z. mobilis* (B). Bacteria from late exponential phase (10 mg. dry wt) were harvested aseptically and suspended in 10 ml. sterile 67 mM phosphate buffer (pH 6.8) in 50 ml. conical flasks under N₂, with and without 33 mM-MgCl₂. Flasks were removed at intervals, bacteria centrifuged down and extinction of the supernatant fluid at 260 nm. measured. Bacteria were fractionated into cold 0.7 N-HClO₄-soluble nucleotide pool and RNA, and these were estimated as described in Methods. Results are expressed as % of dry wt bacteria, with yeast RNA as standard. RNA content: ■, in presence of MgCl₂; □, in absence of MgCl₂. Cold acid-soluble nucleotide pool: ●, in presence of MgCl₂; ○, in absence of MgCl₂. 260 nm.-absorbing material: ▲, in presence of MgCl₂; △, in absence of MgCl₂.

The RNA content of *Zymomonas mobilis* in late exponential phase (average 22 % of dry wt) was very similar to that of *Z. anaerobia*, but marked differences in the rate of RNA degradation were observed for *Z. mobilis*. After anaerobic starvation for 24 hr the RNA content had decreased from 22 % to 11 %; this degradation was prevented by 33 mM-MgCl₂ (Fig. 1B). Degradation of RNA was accompanied by a release of 260 nm.-absorbing material into the medium, while the cold-acid-soluble nucleotide pool remained constant. This initial rapid rate of RNA breakdown then declined and for the next 150 hr was about the same as the rate of breakdown in the presence of MgCl₂. The initial rapid release of 260 nm.-absorbing material in the first 24 hr appeared to be followed by a slow re-utilization during the succeeding 50 hr, an effect never observed with *Z. anaerobia*. MgCl₂ suppressed this release of nucleotides.

Intracellular level of ATP during growth. During growth, the intracellular ATP

content increased as shown in Fig. 2, until the last generation of growth was reached, when a rapid and exponential decrease in ATP began and continued after the glucose in the medium had been exhausted. This decrease was consistently observed in several experiments. In some cases it could be correlated with a decrease in growth rate indicating the approach to the stationary phase, but in other experiments exponential growth was still occurring when the decrease in ATP began.

Effect of addition of substrates on endogenous ATP levels. Within 2 hr of addition of a pulse of glucose (final concentration 2.5 mM) to a suspension of *Zymomonas anaerobia* which had been starved for 25 hr under oxygen-free N_2 , the glucose had

Fig. 2. Intracellular ATP during growth and short-term starvation in growth medium. *Zymomonas anaerobia* was grown anaerobically at 20° and samples were removed aseptically at intervals for measurement of growth (●) and glucose concentration (▲) and extraction of ATP. ■, ATP content (μg./ml. culture).

entirely disappeared from the supernatant fluid (Fig. 3). The concentration of ATP within the cell reflected closely the pattern of glucose utilization. There was a bi-phasic formation of ethanol, the inflexion point (2.1 μmoles) corresponding to 1 mole ethanol/mole glucose added. There then followed a second phase of ethanol formation reaching a maximum with $4.5/2.2 = 2$ moles ethanol formed/mole glucose added. The subsequent loss of ethanol from the supernatant fluid was most probably due to volatilization. In a similar experiment in which glucose was replaced by pyruvate there was no detectable ATP formation, thus confirming that decarboxylation of pyruvate was not an energy-yielding reaction. To test whether the organism can obtain energy from ethanol, the effect of a pulse of ethanol on the ATP levels of a 24 hr-starved suspension of *Z. anaerobia* was determined under both aerobic and anaerobic conditions (Fig. 4). The results suggest that even under anaerobic conditions the organism formed small amounts of ATP from ethanol.

Fig. 3. Effect of anaerobic addition of glucose on ATP levels in starved *Zymomonas anaerobia*. Bacteria (265 mg. dry wt) from late exponential phase were harvested aseptically and suspended in 250 ml. sterile 67 mM-phosphate buffer (pH 6.8) and oxygen-free N_2 bubbled through the suspension. After starvation for 22 hr 625 μ mole glucose were added anaerobically (at point shown by arrow) and samples removed at intervals for assay of ATP (○) μ g. ATP/mg. dry wt of organisms. The culture supernatant fluids were analysed for glucose (▲) and ethanol (■).

Fig. 4. Effect of addition of ethanol, both aerobically and anaerobically, on ATP levels in starved *Zymomonas anaerobia*. Conditions were as for Fig. 3, except that two flasks were set up, one bubbled with air, the other with oxygen-free N_2 . After 24-hr starvation (at the point shown by an arrow) 1 m-mole ethanol was added to each flask (without introduction of air), and samples removed at intervals. Organisms were harvested by centrifugation and ATP extracted from them. The supernatant fluids were analysed for ethanol concentration: ●, aerobic; ▲, anaerobic. ATP pool (μ g. ATP/mg. dry wt); ○, aerobic; △, anaerobic.

*Effect of starvation on ability of Zymomonas anaerobia to
produce ATP from glucose*

The ability to metabolize glucose, and to form ATP from it, remained unimpaired after 143 hr starvation (Fig. 5). In another experiment the starvation was extended to 15 days, after which time the ability to metabolize glucose was impaired, and only low amounts of ATP were formed (maximum, 0.6 $\mu\text{g./mg. dry wt.}$). Bacteria starved for 15 days took 30 hr to metabolize 1 m-mole of glucose as compared with 1 to 2 hr after starvation for 143 hr.

Fig. 5. Effect of extended starvation on the ability of *Zymomonas anaerobia* to produce ATP from glucose anaerobically. Conditions were as for Fig. 3, except that three flasks were set up, each containing 0.4 mg. dry wt organism/ml. and each starved for different periods. After starvation, 1 m-mole glucose was added anaerobically, and samples removed at intervals. Organisms were collected by centrifugation and ATP extracted. The supernatant fluids were analysed for glucose. Solid symbols represent glucose, open symbols ATP content ($\mu\text{g./mg. dry wt.}$), for 23 hr (circles); 47 hr (triangles) and 143 hr (squares) starvation.

*Relationship between viability of Zymomonas anaerobia and intracellular
concentrations of RNA and ATP*

Viability decreased exponentially as soon as starvation began, as might be expected since *Zymomonas anaerobia* contains no energy reserves to protect against starvation. The decrease in viability was accompanied by an exponential decrease in intracellular ATP which was most marked during the initial 6 hr. During longer periods of starvation Mg^{2+} , which prevented degradation of RNA (Fig. 1), seemed to protect the populations. After 175 hr starvation in the presence of 33 mM- MgCl_2 , the population was 22 % viable as compared with only 3 % in the absence of MgCl_2 (Fig. 6). Mg^{2+} did not have a significant effect on the ATP content of the organisms.

An experiment was made to study the effect on ATP amounts of adding glucose to a culture of low viability. Viability was followed as a function of time; after 180 hr, when the viability had decreased to less than 3 %, glucose was added anaerobically (final concentration, 5 mM). The rates of disappearance of glucose and formation and disappearance of ATP were very similar to those shown in Fig. 5. It is apparent that the ability to produce ATP in response to glucose is not lost on prolonged starvation and is, therefore, not related to viability as measured by slide culture.

Fig. 6. Effect of prolonged starvation on intracellular ATP levels and viability of *Zymomonas anaerobia* with and without Mg^{2+} . Conditions were as for Fig. 3. Δ , Intracellular ATP concentration and $\blacktriangle$, viability in the absence of $MgCl_2$; $\circ$, ATP concentration and $\bullet$, viability in the presence of 33 mM- $MgCl_2$.

DISCUSSION

A study of the intracellular constituents of *Zymomonas anaerobia* and *Z. mobilis* which are degraded on starvation suggests that, in both organisms, the only constituent which is significantly broken down is RNA. Breakdown of RNA seems to be a common (though by no means universal) response of bacteria to starvation. Thus *Aerobacter aerogenes* (Strange, Dark & Ness, 1961), *Pseudomonas aeruginosa* (Gronlund & Campbell, 1965), *Escherichia coli* (Dawes & Ribbons, 1965), *Sarcina lutea* (Burleigh & Dawes, 1967) and *Streptococcus lactis* (Thomas, 1968) all degrade RNA during starvation. Anaerobic organisms would thus not seem to differ from aerobes in this respect. In all cases, the breakdown of RNA is prevented by Mg^{2+} , which is well known as a ribosome-stabilizing agent (Bowen, Dagley & Sykes, 1959; Wade, 1961). In the case of *P. aeruginosa*, Gronlund & Campbell (1965) have shown that the ribosomes are degraded. Fig. 6 shows that Mg^{2+} ions exert a considerable protective effect against loss of viability in *Z. anaerobia*. In this respect, the organism resembles *A. aerogenes* (Strange & Shon, 1964; Strange & Hunter, 1967) and *S. lactis* (Thomas & Batt, 1968) rather than *E. coli* (Dawes & Ribbons, 1965) or *S. lutea* (Burleigh & Dawes, 1967), where survival is not prolonged by Mg^{2+} ions. Ethanol, unlike pyruvate, leads to the formation of small amounts of ATP, even under anaerobic conditions. This observation suggests that the metabolism of *Z. anaerobia* may be somewhat

more complex than has hitherto been envisaged. The higher level of ATP formed in aerated suspensions is in keeping with the ability of washed cell suspensions of anaerobically grown *Z. anaerobia* to oxidize ethanol without a lag (P. J. Large, unpublished observation). This may indicate that ethanol can be metabolized further by *Z. anaerobia* and that this metabolism may be energy-yielding. Belaïch & Senez (1965) found that *Z. mobilis* would oxidize ethanol, but suggested that the process was not energy-yielding.

While the *ability to produce* ATP is ultimately impaired by starvation, the actual intracellular amounts of ATP seem to be correlated with viability of the cells (Fig. 6). Although several workers (e.g. Cole *et al.* 1967 with *Escherichia coli*) have studied the effects of starvation on ATP levels, the only work of this kind that we are aware of in which viabilities were measured is that of Strange, Wade & Dark (1963). In contrast to the present observations, these workers were unable to detect any correlation between intracellular ATP content of *Aerobacter aerogenes* and viability, the ATP amounts reflecting largely the oxygen tension and solute concentration. A higher ATP content in *E. coli* starved aerobically was also observed by Cole *et al.* (1967). We have been unable to discover any correlation between viability and ability to produce ATP from glucose in *Zymomonas anaerobia*.

The marked decrease in intracellular ATP in the last generation before growth on glucose ceased was observed in several experiments. This phenomenon does not seem to have been explicitly reported by other workers. Forrest & Walker (1965), in *Streptococcus faecalis* growing on glucose, only observed a decline in ATP amounts when the stationary phase had been reached. Cole *et al.* (1967) measured the ATP pool in *Escherichia coli* under various conditions. When the organisms were grown anaerobically an apparent decline in ATP content did occur; in defined medium containing glucose the amount of ATP decreased abruptly in the stationary phase, while in glucose+yeast-extract medium a slow decrease in ATP content occurred in the last half-generation before growth ceased. We suggest that the decrease in ATP content in *Zymomonas anaerobia* during growth on glucose (which is here behaving as energy source) is an indication that the organisms are becoming energy limited. In cultural conditions where growth is limited by some other constituent of the medium the ATP pool might be expected to behave differently.

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