JOURNAL OF BACTERIOLOGY, June, 1965
Vol. 89, No. 6

Printed in U.S.A.

Synthesis of Reserve Materials for Endogenous Metabolism in *Streptococcus faecalis*

W. W. FORREST AND D. J. WALKER

Division of Biochemistry and General Nutrition, Commonwealth Scientific and Industrial Research Organization, University of Adelaide, Adelaide, South Australia

Received for publication 18 November 1964

ABSTRACT

FORREST, W. W. (University of Adelaide, Adelaide, South Australia), AND D. J. WALKER. Synthesis of reserve materials for endogenous metabolism in *Streptococcus faecalis*. J. Bacteriol. **89**:1448–1452. 1965.—The growth curve of *Streptococcus faecalis* in batch culture with limited energy source shows an initial portion of exponential growth where the growth yield coefficient $Y_{glucose}$ is 32, followed by linear growth where $Y_{glucose}$ is 21. Endogenous metabolism is correlated with maintenance of glycolytic activity, and there is a marked change in the glycolytic activity of cells harvested under these conditions; however, endogenous metabolism cannot be detected in either case by measurement of the adenosine triphosphate (ATP) pool in the organisms. Cells harvested after growth with excess energy source exhibit endogenous metabolism, which is correlated with a much higher concentration of ATP in the organisms than occurs in the cells grown with limited energy source.

It has been shown that *Streptococcus faecalis* may exhibit a substantial anaerobic endogenous metabolism after growth on media containing excess energy source. This endogenous metabolism appears to provide energy which the organisms use to maintain a constant level of activity in the complex of glycolytic enzymes for several hours after the removal of exogenous energy sources (Forrest and Walker, 1963). Cells grown exponentially with the energy source limiting growth have no detectable endogenous metabolism and no period of constant glycolytic activity (Forrest and Walker, *in press*).

However, cells grown on complex media with excess energy source show this initial period of exponential growth, with a later period of linear growth. The experiments of Bauchop and Elsden (1960) on the growth of microorganisms in relation to their energy supply show that the growth of *S. faecalis* is not a linear function of substrate concentration down to zero substrate concentration; this atypical behavior suggests a change in the pattern of growth, with the possibility that synthesis of reserve materials is taking place. Anaerobic endogenous metabolism has been little studied (Dawes and Ribbons, 1962, 1964), but Hungate (1963) has shown that, during the later stages of growth, *Ruminococcus albus* lays down storage polysaccharide which is degraded after growth ceases. Senez and Belaich (1964) have shown by calorimetric studies that *Escherichia coli*, which lays down glycogen during growth, exhibits a large anaerobic endogenous metabolism for long periods after growth has ceased.

The experiments reported have, therefore, been carried out to determine the conditions of growth necessary to allow *S. faecalis* to synthesize reserve material. The experimental approach was necessarily rather indirect, as a direct assay for endogenous reserves in this organism has not yet been developed.

MATERIALS AND METHODS

Growth of organisms. For measurements of growth yield, a series of culture tubes was prepared. All tubes contained 25 ml of a complex medium with a final concentration of 2% dried yeast extract (Difco), 2% dried peptone (Difco), and 2% sodium citrate; *p*H of the medium was 6.0. To each tube was added as energy source a different concentration of glucose covering the range of 0 to 2%. A 10% glucose solution was sterilized separately from the medium; the required volume of this solution was added to 20 ml of initially 2.5% medium in each tube, and the volume in the tube was adjusted to 25 ml with sterile water. Anaerobic precautions were not observed during growth. The course of growth was followed by nephelometric measurement. In the tubes with glucose concentrations up to 25 μmoles/ml, growth was considered complete when no further increase in nephelometric reading occurred after 2 hr. In those with higher concentrations of glucose, where the last part of growth occurred very slowly,

growth was allowed to continue overnight. After this period, the cells were harvested by centrifugation, and dry weight determinations were carried out.

For adenosine triphosphate (ATP) assays during growth, 600-ml samples of this 2% complex medium with 2% glucose as energy source were taken. After inoculation, 20-ml samples were taken during the course of growth for ATP assays. Replicate experiments were carried out to determine the effect of anaerobiosis. In one set, the medium was deaerated, reduced with 0.05% sodium thioglycolate, and gassed during growth with a slow stream of oxygen-free nitrogen. Parallel experiments were carried out under static aerobic conditions, as in normal batch culture.

Subcultures of the organism (*S. faecalis* ATCC 4083) were maintained on the complex medium with 2% glucose added. All growth and incubation of cells was done at 37 C.

Preparation of washed suspensions. Cells were harvested as soon as possible after the cessation of growth, washed twice in deaerated 0.1 M potassium phosphate buffer (*p*H 6.0), and suspended in the same buffer under an atmosphere of nitrogen.

Determination of glycolytic activity. Samples of suspensions containing 5 to 10 mg of cells were centrifuged. This process yielded a cell paste which was suspended in 10 ml of 0.01 M phosphate buffer (*p*H 6.0). A 50-mg amount of glucose was added as substrate, and the rate of production of hydrogen ion at *p*H 6.0 and 37 C was followed by titration with 0.1 M NaOH.

Dry-weight determinations. Dry-weight determinations were carried out by measuring the optical density of suitably diluted suspensions at 650 mμ and referring to a standard curve of dry weight against optical density.

ATP assays. The method used for ATP assays was a modification of that of Strehler and Totter (1952). A 100-mg amount of dried firefly lanterns (Sigma Chemical Co., St. Louis, Mo.) was extracted in a manual homogenizer, which was cooled in an ice bath with 5 ml of 0.025 M arsenate-sulfate buffer (*p*H 7.4), and was then clarified by centrifugation at 60,000 *g* × for 30 min in the cold. The preparation was made fresh daily and kept in an ice bath.

The assay cuvette contained initially 1 ml of bacterial extract and 2 ml of 0.01 M phosphate buffer (*p*H 7.4) containing 0.75 mg/ml of $MgSO_4$. Exactly 0.1 ml of the extract of firefly tails was then added rapidly from a syringe, and the light output was measured 15 sec after mixing. Adenosine diphosphate (ADP) was slowly converted to ATP by the myokinase present in the extract of firefly tails, but the conversion had a half-time of several minutes under these conditions. By reading the light output as soon as possible after mixing, interference from ADP became insignificant.

The measurement of light intensity was made with a photomultiplier tube (type 27MI; Edison Swan, Ltd., London, England); the photocurrent from the tube was monitored with a strip-chart recorder (model 80; Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio).

The preparation of extracts of ATP from the bacteria was found to be a critical part of the assay. Both the perchloric acid extraction commonly used and extraction with boiling water have been criticized (Beutler and Baluda, 1964), and our experiments confirmed these criticisms. The method found effective with this organism was as follows. A volume of cell suspension containing about 10 μg of ATP was centrifuged, and the cell paste obtained was suspended in 0.5 ml of 0.3 M H_2SO_4. This suspension was allowed to stand for 30 min at room temperature; the extract was neutralized with NaOH, and the volume was made up to 10 ml. The extract was then centrifuged, and 1 ml of the supernatant fluid was assayed.

Standard ATP solutions containing the electrolytes present in the bacterial extracts were assayed with daily samples.

RESULTS

There is a pronounced change in the properties of the organisms during growth. The inoculum used was less than 1% of the final cell yield and, for the first five generations of growth, was exponential; above this point there was an inflection in the growth curve, with linear growth taking place beyond this for only one or two generations. The *p*H of the medium at the point of inflection was 5.7. The behavior of the ATP pool during growth reflects this marked change (Fig. 1). In this experiment, excess energy source was added so that the complete growth curve could be observed.

Early in growth, the size of the ATP pool increased linearly, though the cells grew exponentially. At the point of inflection, when 6 μmoles of glucose per ml of medium had been consumed by the organisms, there was a sharp change in the rate of increase of the ATP pool; the cell density and ATP pool both increased linearly from this

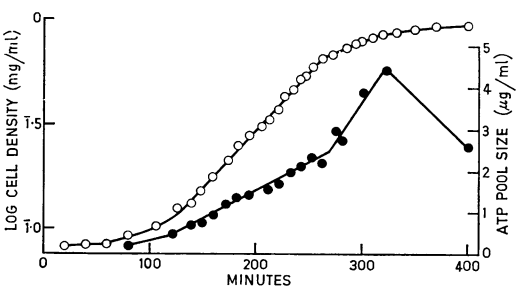

FIG. 1. *AIP pool during growth on complex medium containing 2% glucose. Symbols:* ○, *log cell density (measured nephelometrically);* ●, *ATP pool size.*

point until the size of the ATP pool reached a maximum and began to diminish again at the end of growth. Whether or not anaerobiosis was maintained, the growth rate and ATP pool size were unaffected. The change from exponential to linear growth in both cases took place at the same point, and the growth yield was not significantly different.

Similar transitions occurred in the growth yield of the cells (Fig. 2). In these experiments, growth was limited by energy source, so that the cultures with little energy source underwent exponential growth only, and those with more than 6 μmoles/ml of glucose had an initial period of exponential growth followed by linear growth. The growth yield coefficient $Y_{glucose}$ (Bauchop and Elsden, 1960) during exponential growth was 32 g (dry weight) of cells per mole of substrate. This is in agreement with a value of 30 for $Y_{glucose}$ calculated from previously reported yields during exponential growth of this strain of *S. faecalis* on semidefined medium (Forrest and Walker, 1964). The fermentation of glucose during exponential growth is not homolactic; up to 50% of steam-volatile acids are produced (Forrest, Walker, and Hopgood, 1961). Over the linear part of the growth curve, $Y_{glucose}$ fell to 21; Bauchop and Elsden (1960), all of whose determinations on *S. faecalis* seem to have been made in this range, reported similar results for growth on complex medium, where the fermentation of glucose was homolactic. The complex medium contained carbohydrates which allowed growth in the absence of glucose. The growth so produced gave the point at zero glucose concentration in Fig. 2.

The glycolytic activity of washed suspensions of the cells grown in these experiments also

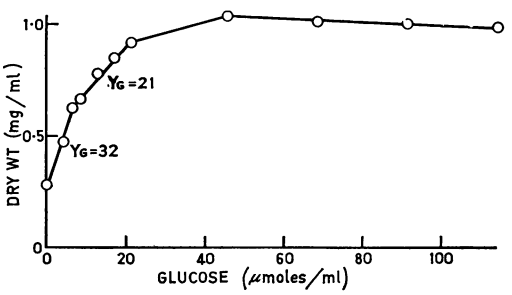

FIG. 2. *Growth yields on complex medium, with growth limited by energy source. Different amounts of glucose were added to samples of complex medium, and growth was measured nephelometrically. When growth was completed, cells were harvested, washed, and resuspended in phosphate buffer. Cell yield was determined by measurement of the optical density of this suspension at 650 mμ.*

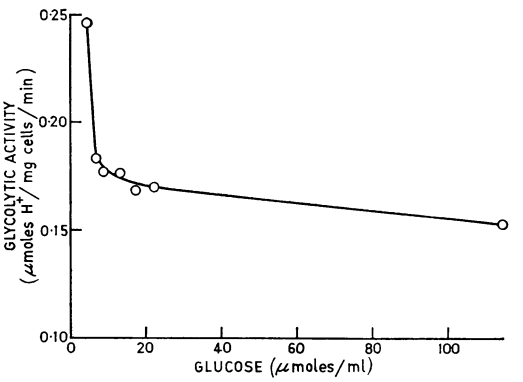

FIG. 3. *Glycolytic activity of cells after growth on complex medium. A 10-ml amount of medium from growth experiments described in Fig. 2 was centrifuged, cells were suspended in 10 ml of 0.01 M phosphate buffer, 50 mg of glucose were added, and the pH was maintained at 6.0 by titration with 0.1 M NaOH at 37 C.*

showed a change at the point of inflection in the growth curve, falling to the level characteristic of cells grown with excess energy source (Forrest and Walker, 1963, 1964) at the point at which linear growth began (Fig. 3). It should be noted that cells grown either exponentially or linearly have a glycolytic activity in washed suspension of only about one-fifth that observed during growth (Forrest and Walker, 1964), so that this finding does not imply the same quantitative relationships in growing cells.

The process of endogenous metabolism is correlated with a constant ATP pool in a washed suspension of cells. The energy required for maintenance appears to be coupled by ATP to the maintenance reactions (Forrest and Walker, 1964); therefore, the absence of any appreciable ATP pool in the cells is a clear indication that no energy of maintenance is being supplied to the cells. Cells grown with excess glucose maintain a constant ATP pool of 0.7 to 0.8 μg of ATP per mg of cells for several hours during the period of endogenous metabolism, but cells grown with growth limited by energy source did not exhibit this constant ATP pool or a constant level of glycolytic activity. This behavior was found both when the cells were harvested immediately after growth had ceased (Fig. 4 and 5) and, under conditions strictly comparable with cells grown on medium containing 2% glucose, when harvested from the medium 17 hr after inoculation.

Similarly, it had been suggested that the fall in the concentration of ATP at the end of linear growth (Fig. 1) might be due to the synthesis of reserve materials, which would require a supply

of energy. A similar experiment to that of Fig. 1 was, therefore, carried out. The ATP pool was measured during growth, and the cells grown in this manner were harvested 150 min after the fall in the concentration of ATP began. At this point, the organisms had consumed 27 μmoles/ml of glucose. A washed suspension of these cells was prepared, and the pool of ATP in the suspension was determined in the same way as the experiment of Fig. 5. The results were comparable with those shown in Fig. 5 for glucose-limited cells. No detectable endogenous metabolism was found, either in cells grown anaerobically or under static aerobic conditions.

DISCUSSION

The results reported show that the synthesis of cellular material by *S. faecalis* occurs in three stages, and that the organisms have distinctly different properties in each of these stages.

The first stage is exponential growth; in this stage, the products of glycolysis include steam-volatile acids and the growth yield coefficient $Y_{glucose}$ is 32, suggesting that 3 moles of ATP are produced per mole of glucose fermented. Homolactic fermentation by the Embden-Meyerhof pathway involves the production of 2 moles of pyruvate and 2 moles of ATP per mole of glucose; this pyruvate is then fermented to lactate with no further production of ATP. However, some of the pyruvate may, instead, undergo dismutation with the production of formic acid and acetic acid, CO_2, and up to another mole of ATP per mole of pyruvate.

The second stage is linear growth; in this stage $Y_{glucose}$ is 21, suggesting that 2 moles of ATP are produced per mole of glucose fermented, and that fermentation is homolactic.

The third stage is a period of synthesis of reserve materials without a significant amount of growth where it is meaningless to assign a value for $Y_{glucose}$.

The transition between exponential and linear growth has been exhaustively discussed (Lamanna and Mallette, 1959). In general, the transition is due to limitation of some constituent in the medium. In aerobic organisms, lack of oxygen is a common cause, but, in the present case, the effect is not an artifact caused by oxygen affecting the fermentation pathway. Bauchop and Elsden (1960) maintained strict anaerobiosis in their experiments; our results agree closely with theirs, and the course of fermentation is unaffected by the presence or absence of air. This transition is accompanied not only by a change in the calculated rate of production of ATP, but also by a sharp inflection in the measured rate of increase in the ATP pool size, showing directly that there is a pronounced change in the metabolism and energy requirements of the organisms at this point.

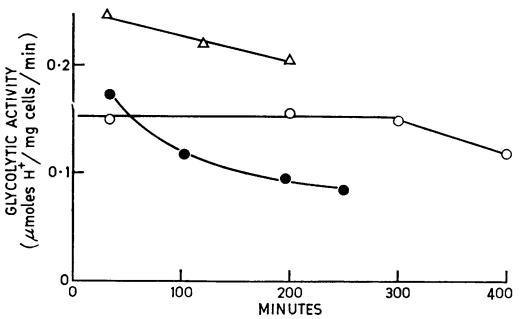

FIG. 4. *Time course of glycolytic activity of cells in washed suspension after growth on complex medium. Cells were harvested immediately after growth ceased, washed twice with deaerated 0.1 M phosphate buffer (pH 6.0), and suspended in the same buffer under nitrogen at a cell density of 1 mg/ml. Samples (5 ml) were taken, the cells were harvested and resuspended in 0.01 M phosphate buffer, and glycolytic activity was measured as described in Fig. 3. Symbols for glucose concentration in growth medium: △, 4.4 μmoles/ml; ●, 19.5 μmoles/ml; ○, 110 μmoles/ml.*

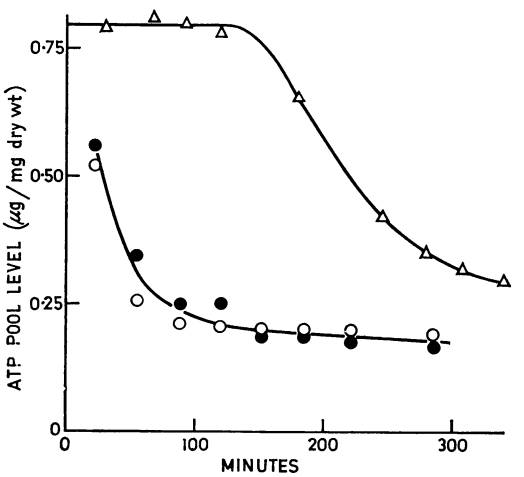

FIG. 5. *ATP pool level of cells in washed suspension after growth on complex medium. Washed suspensions were prepared as described in Fig. 4. Symbols for glucose concentration in growth medium: ●, 4.4 μmoles/ml; ○, 19.5 μmoles/ml; △, 110 μmoles/ml.*

It has been shown that, when *S. faecalis* is grown under the conditions used in these experiments, the rate of catabolism of glucose by unit mass of cells remains constant during exponential

growth, and then begins to fall rapidly at the point at which linear growth begins (Forrest and Walker, 1964). When the cells are harvested and suspended in buffer, their capacity for glycolysis falls to a rate of about one-fifth that observed during growth, but the difference in glycolytic activity between cells harvested during exponential or linear growth is more pronounced. It seems, therefore, that the difference can not be due simply to the immediate effect of the environment in which the cells are placed; in both cases, glycolysis must be greatly repressed, and the repression is less in cells which have not begun linear growth.

The measurements of the level of the ATP pool in washed suspensions indicate that, despite these changes in the metabolic activity of the cells, elaboration of reserves for endogenous metabolism does not occur to any measurable extent until after the period of linear growth. After the end of linear growth, the catabolism of the substrate gives rise to ATP which cannot be used directly for growth. The medium at this point is exhausted of growth factors, though it still contains amino acids. It has been shown that *S. faecalis* in a washed suspension supplied only with amino acids and energy source can make use of the ATP generated by glycolysis for synthesis, without growth, of materials related to the maintenance of glycolytic activity (Walker and Forrest, 1964; Forrest and Walker, *in press*).

Under the conditions of the present experiment, there can be ample excess glucose to incorporate all the amino acids still present in the medium into cellular reserve materials. Stoichiometrically, only about 0.5% glucose can be used by the organisms as an energy source for growth on this medium; therefore, in the medium containing 2% glucose, a large excess of energy source becomes available at the end of growth. Energetically, glycolysis by cells in a washed suspension generates ATP rapidly enough to maintain a pool level about 10 times as great as that which occurs during endogenous metabolism (Forrest and Walker, *in press*); thus, a large ATP pool is available to supply energy for synthetic reactions.

It seems, therefore, that synthesis of reserves occurs after the end of linear growth, without any appreciable increase in cell density. The converse experiment of measuring the decrease in optical density of a cell suspension during endogenous metabolism gives a loss of about 5%. It might be expected that the amount of reserves produced would be larger than this figure. Very large reserves have been reported in other organisms (Hungate, 1963; Dawes and Ribbons, 1962), and calculations based on the amount of heat generated by endogenous metabolism (Forrest and Walker, 1963) indicate that a large fraction of the cellular material of cells grown with excess energy source would be reserves.

However, the energy-producing reactions of endogenous metabolism and the reserve material have not been characterized, so that more definite conclusions can not yet be reached.

ACKNOWLEDGMENT

We thank P. R. Monk for his assistance during this investigation.

LITERATURE CITED

BAUCHOP, T., AND S. R. ELSDEN. 1960. The growth of micro-organisms in relation to their energy supply. J. Gen. Microbiol. **23**:457–469.

BEUTLER, E., AND M. C. BALUDA. 1964. Simplified determination of blood adenosine triphosphate using the firefly system. Blood **23**:688–698.

DAWES, E. A., AND D. W. RIBBONS. 1962. The endogenous metabolism of microorganisms. Ann. Rev. Microbiol. **16**:241–264.

DAWES, E. A., AND D. W. RIBBONS. 1964. Some aspects of the endogenous metabolism of bacteria. Bacteriol. Rev. **28**:126–149.

FORREST, W. W., AND D. J. WALKER. 1963. Calorimetric measurements of energy of maintenance of *Streptococcus faecalis*. Biochem. Biophys. Res. Commun. **13**:217–222.

FORREST, W. W., AND D. J. WALKER. 1964. Change in entropy during bacterial metabolism. Nature **201**:49–52.

FORREST, W. W., D. J. WALKER, AND M. F. HOPGOOD. 1961. Enthalpy changes associated with the lactic fermentation of glucose. J. Bacteriol. **82**:685–690.

HUNGATE, R. E. 1963. Polysaccharide storage and growth efficiency in *Ruminococcus albus*. J. Bacteriol. **86**:848–854.

LAMANNA, C., AND M. F. MALLETTE. 1959. Basic bacteriology, p. 344. The Williams & Wilkins Co., Baltimore.

SENEZ, J. C., AND J. P. BELAICH. 1964. Étude microcalorimétrique de la croissance bactérieene et du contrôle de l'activité métabolique par le phosphate. *In* Colloque international sur la régulation des activités cellulairs chez les microorganismes. Centre National de la Recherche Scientifique, Paris.

STREHLER, B. L., AND J. R. TOTTER. 1952. Firefly luminescence in the study of energy transfer mechanisms I. Substrate and enzyme determination. Arch. Biochem. Biophys. **40**:28–41.

WALKER, D. J., AND W. W. FORREST. 1964. Anaerobic endogenous metabolism in *Streptococcus faecalis*. J. Bacteriol. **87**:256–262.