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Rapid Determination of Microbial Batch Culture Stage

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Bacterial populations with known physiological characteristics are required for many experiments, for example, to determine the efficiency of ATP extraction from sediments and soils (WILDISH et al. 1979) or in nutritional studies of suspension feeding animals (WILDISH et al., in preparation). One method of characterising the microbial population is to determine its adenylate energy charge (EC) ratio. The characteristic growth stages of a carbon limited, aerobically grown batch culture of Escherichia coli (CHAPMAN et al. 1971) are: log where the ATP per cell is high and EC = 0.8, stationary where the ADP per cell is high and EC = 0.8 - 0.5, senescent where the AMP concentration is high and EC = 0.5 - 0.2. The determination of EC ratios is, however, slow and relatively expensive for routine use. The purpose of this paper is to present results which form the basis of a rapid method for estimating the physiological status of bacterial cultures.

The firefly bioluminescence assay reaction (LEE et al. 1971) for the determination of ATP involves formulation of an enzyme substrate complex and is specific to ATP:

$$ATP+LH_2+E \xrightarrow{Mg^{++}} E\text{-}LH_2\text{-}AMP+P\text{-}P \quad \ldots\ldots\ldots\ldots 1,$$

$$E\text{-}LH_2\text{-}AMP+O_2 \longrightarrow E+product+CO_2+AMP+light \quad \ldots 2$$

Crude preparations of firefly lantern extracts contain traces of enzymes such as adenylate, nucleoside diphosphate pyruvate and pyruvate kinases (LUNDIN & THORE, 1975) able to convert ADP or non-adenylate di- or triphosphate nucleotides to ATP. Reactions 1 and 2 are completed within 3 seconds and because of the consumption of ATP by luciferase ADP or non-adenylate nucleotides are phosphorylated to ATP. The further ATP produced is then utilized to give additional light by reactions 1 and 2 which characteristically prolongs the light emission decay curve.

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EXPERIMENTAL

BATCH CULTURE OF BACTERIA

Cultures were grown aerobically at 27±2°C in conical flasks or fermenters containing 1 litre of Oxoid CMI medium (1.3 g/l) in seawater. Numbers of viable cells per ml was estimated by the spread plate technique on 2% agar (Oxoid No. 3) made up with CMI medium. The following bacteria were used: Staphlyoccus aureus (National Culture Industrial Bacteria No. 6571), a marine Pseudomonas sp. (National Culture Marine Bacteria No. 130) and Serratia marcescens (National Culture Marine Bacteria No. 4).

ATP ANALYSIS

Extraction was made by aseptic transfer of 0.1 ml of culture medium directly into 5 ml of boiling

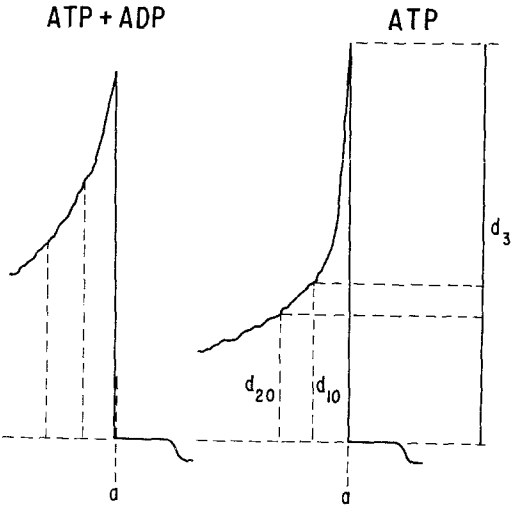

Fig 1. Time course of light emission for ATP (17.5µg/1) and ATP (17.5µg/1)+ ADP (180µg/1) injected at point a

Tris buffer (0.2 M Tris (hydroxymethyl) aminomethane adjusted to pH 7.8 with (HCl.). All transfers were made within 5 seconds to avoid EC ratio changes which may occur due to stress (NIVEN et al. 1977). Following 2 minutes boiling the extract was cooled on ice. ATP standards (sodium salt of adenosine 5' - triphosphate) were made up in the same batch of 0.2 M Tris buffer and stored, with the extracts, at -20°C until needed.

Firefly lantern extract, FLE50 from Sigma Chemical Co., St. Louis, Missouri, was rehydrated with 10 ml of sterile distilled water: the resultant solution

contains 0.1 M magnesium sulphate, 0.1 M potassium arsenate, luciferin-luciferase plus contaminant enzymes obtained from 50 mg of firefly tails at a pH of 7.4. After ageing, the solution was centrifuged and stored in the dark until used.

The instrument used to measure light emission was a modified Farrand fluormeter with a hand-operated injector (WILDISH 1976) linked to a fast-response recorder (0.5 seconds full scale deflection) although any commercial ATP photometer which permits stray light-free recording from the moment the reactants are mixed would be suitable. The reaction was begun by

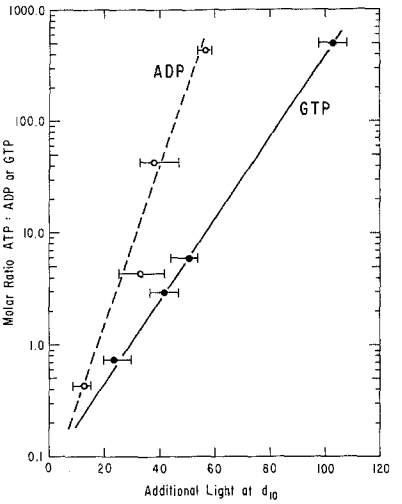

Figure 2. Effect of ATP:ADP and ATP:GTP in different molar ratios on the percentage additional light at d_{10}. Mean and range of 4-6 determinations.

injecting 0.2 ml of standard ATP, or extract, solution into 0.2 ml of firefly lantern extract at a speed which ensured rapid mixing. The final pH of the reactant solution was pH 7.4±0.2 and assay temperature was 20±2 C. Typical recorder chart outputs are shown in Fig. 1. Pen deflection as arbitrary counts at peak height (which occurs 2 to 3 seconds after injection), at 10 seconds (d_{10}) and 20 seconds (d_{20}) after injection were multiplied by a sensitivity factor to give counts which after correction for background light emission were linearly related to ATP concentration on logarithmic co-ordinates. Additional light at d_{10} was calculated as a percentage of the arbitrary counts at peak height:

$$\frac{d_{10} \times 100}{d}_{\text{peak unknown}} \quad - \quad \frac{d_{10} \times 100}{d}_{\text{peak ATP standard}}$$

ADP AND GTP STANDARD ADDITION

Various concentrations of ATP:ADP and ATP:GTP were injected into firefly lantern extract. Adenosine 5' - diphosphate and guanosine 5' - triphosphate were obtained from Sigma and hydrated in Tris buffer of pH 7.8.

RESULTS

The percentage additional light at d_{10} is linearly related to a semi-logarithmic plot of the molar ratio of ATP:ADP standards (Fig. 2.). Other non-adenylate nucleotides such as GTP also interfere with a more marked effect than ADP above a molar ratio of 0.1. It is not possible to separate light emission due to ADP and GTP by comparing the ratio d_{10}/d_{20}, although this might be possible with other non-adenylate triphosphates which show a longer delay in attainment of the maximum additional light (WILDISH 1976).

Typical results with batch cultures of S. aureus (Fig. 3A) show a characteristic pattern. The ATP concentration reaches a peak in early stationary phase. This is followed by a rapid decline to a plateau level. Data shown in Fig. 3B suggests that loss of ATP was due to quantitative conversion to ADP. In late stationary phase there is a further drop in ATP partly due to further intracellular conversion of ATP to ADP, AMP and non-adenylates and to the effect of AMP in inhibiting ATP determination by peak height methods. The concentration of ATP per cell (µg ATP/cell) in S. aureus was 1×10^{-6} in exponential, reaching 4×10^{-6} in early stationary and declining to 8×10^{-8} in the late stationary phase. With cell death in the senescent phase the ATP per viable cell increases again because of the release of intracellular material which is available to other cells.

Reproducibility of the method (as twice the standard of error) for ATP 10 µg/l is ± 4% for both peak and d_{10} measures. Possible systematic errors, not included in this estimate are: non-representative sampling of the culture and differences in speed of transference to the boiling extract solution. Differences between batches of firefly lantern extract (e.g. in amount of adenylate kinase) and variation in pH and temperature between assays would be accounted for by use of ATP standards with each run.

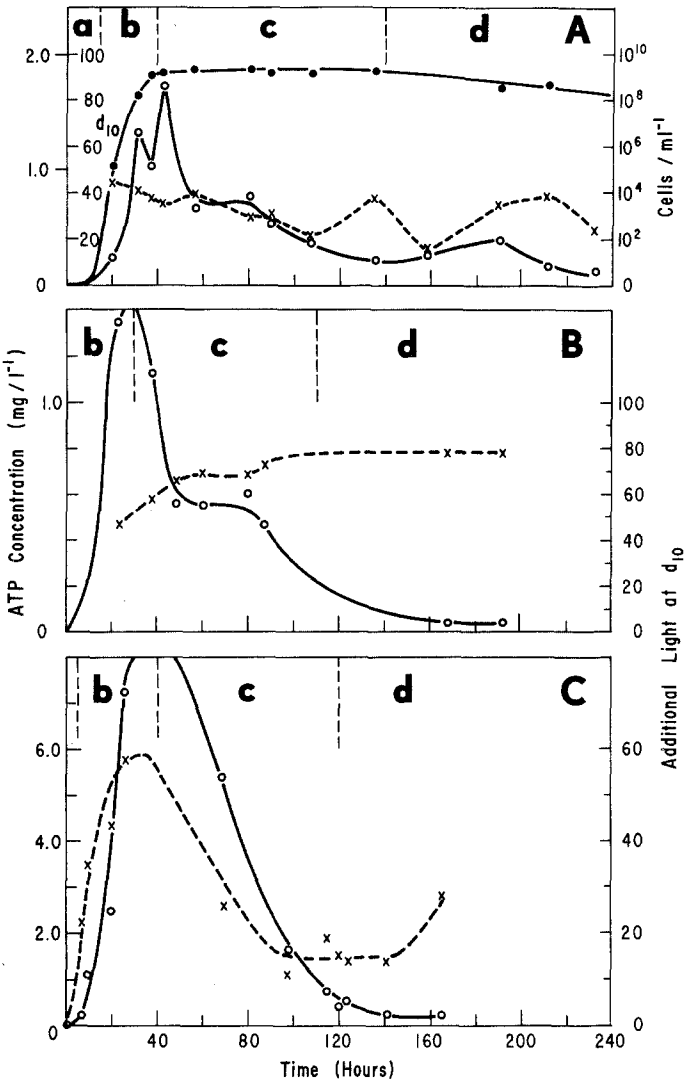

Figure 3A S. aureus; B Pseudomonas sp.; C S. marcescens; a-lag, b-exponential, c-stationary, d-senescence. Solid dots cell numbers, open dots ATP concentration and crosses additional light at d_{10}.

DISCUSSION

The described method allows characterization of the physiological stage of microbial batch cultures sufficient for the environmental purposes outlined in the introductory paragraph. The method is also simpler and less expensive than that used to determine EC ratios.

Previous field or laboratory ATP determinations using Tris extraction, crude firefly lantern extract, and integrated peak area running for longer than 3 seconds after injection may thus incorporate error due to the enzymic conversion of ADP or other non-adenylate nucleotides to ATP. Field recognition of unidentified peaks in the light emission decay curve were made on samples from a pulp polluted, anoxic marine inlet (WILDISH 1976) caused by unidentified contaminants (ADP or non-adenylates) in the extract solution which are enzymatically phosphorylated to ATP. KARL (1977, 1978a) also found interferences of this kind in anoxic water of the Black Sea, which were due to GTP + UTP rather than ADP. The presently described rapid method cannot distinguish between ADP and various non-adenylate nucleotides, although KARL (1978b) has described a selective enzymatic pre-incubation technique to do so.

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