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THE REDOX ENVIRONMENT AND MICROBIAL PHYSIOLOGY

1. THE TRANSITION FROM ANAEROBIOSIS TO AEROBIOSIS IN CONTINUOUS CULTURES OF FACULTATIVE ANAEROBES

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SUMMARY

1. Using continuous culture techniques the transition from anaerobiosis to aerobiosis has been investigated in *Escherichia coli* and *Klebsiella aerogenes*.

2. The use of redox potential and oxygen partial pressure measurements have been compared.

3. Physiological changes in the cells have been examined at different aeration rates, four growth phases can be detected each at characteristic redox potentials. These are: Phase 1. Anaerobiosis, at < 0 mV. Phase 2. Limited aeration at about $+100$ mV. Cytochrome levels are maximal. Phase 3. Aerobiosis between $+200$ and $+300$ mV. Tricarboxylic acid cycle enzyme levels, steady-state ATP pool and growth yield are at their highest. Phase 4. Supra-optimal aeration conditions at $> +300$ mV. All activities are lowered.

4. The reason for these changes is discussed together with the use of redox measurements in bacterial cultures.

INTRODUCTION

Oxygen is perhaps the most important regulator of the chemical make up of microorganisms capable of growing both aerobically and anaerobically. Oxygen can induce: mitochondrial formation in yeast[1-3]; respiratory capacity, cytochrome synthesis and respiratory dehydrogenase enzymes in yeast[4], Staphylococci[5,6], *Escherichia coli*[7,8] and *Pasteurella pestis*[9,10], tricarboxylic acid cycle enzymes in yeast[11], *E. coli*[12], *P. pestis*[10] and *Aerobacter aerogenes*[13]; oxidative phosphorylation in *E. coli*[14] and the biosynthesis of quinones in various organisms[15,16]. At the same time oxygen represses enzymes involved in electron transport to acceptors other than oxygen. These enzymes include nitrate and nitrite reductase[17-19], thiosulphate reductase[20], hydrogenase[20] and a variety of organic electron acceptor reducing enzymes such as fumarate reductase[21], ethanol dehydrogenase[22,23] lactate dehydrogenase[22,24], glycerol dehydrogenase[23] and soluble formate dehydrogenase in several organisms. These changes are now well documented and readily interpreted in batch culture experiments. Limited results have been obtained from continuous culture experi-

ments: for example Moss[25,26] has demonstrated increased cytochrome a_2 levels in both *A. aerogenes* and *E. coli* at low oxygen tensions when oxygen was varied[25,26], HARRISON AND PIRT[27] reported significant changes in metabolic product accumulation as oxygen was increased in continuous cultures of *Klebsiella aerogenes*.

When oxygen is systematically varied it is usually measured in the growth vessel using an oxygen measuring electrode. This device has one major disadvantage in that at low aeration rates when oxygen is limiting one is attempting to measure vanishingly low amounts of dissolved oxygen as it passes from the aerating bubble down a very steep gradient to a biological system having a very high affinity for this substrate.

We came to the conclusion that measurements of redox potential might be a more significant parameter than oxygen measurements in this region. DANIELS *et al.*[28] and NYIRI AND LENGYEL[29] have also examined the possibility of redox measurements as an index of aeration. The latter workers concluded that in their system the E_h was directly related to dissolved oxygen.

This will be examined in more detail in this paper, which will attempt to relate some physiological changes in chemostat cultures of facultative anaerobes to both oxygen tension and E_h.

MATERIALS AND METHODS

Organisms and media

E. coli, strain K12, and *K. aerogenes*, Dartmouth Medical School, U.S.A. strain 203, were maintained on nutrient agar slopes.

The synthetic medium contained per l: 0.54 g KH_2PO_4; 0.22 g KOH; 2.6 g NH_4Cl; 1.3 ml of antifoam (Silicone MS antifoam emulsion RD, Hopkin and Williams); and 5 ml mineral salts solution. The stock solution of mineral salts contained per l: 10 g $MgSO_4 \cdot 7H_2O$; 1 g $MnCl_2 \cdot 4H_2O$; 0.4 g $FeSO_4 \cdot 7H_2O$; and 0.1 g $CaCl_2$. It was stabilised by adjusting the pH to 2.0 with HCl.

The complex medium contained per l: 5 g tryptone (Oxoid); 0.7 g KH_2PO_4; 1.3 ml of antifoam; and 5 ml mineral salts solution. Glucose solution was added to media after separate sterilisation to a final concentration of 4 g/l.

Growth of organisms and preparation of extracts

The organisms were grown in a chemostat, under conditions of carbon-limitation, using equipment described by ELSWORTH *et al.*[1]. A culture volume of 3 l and a dilution rate of 0.16 h^{-1} were used. Organisms were grown at 37° and pH 7.0, and sparged with 1 l of gas per min. During anaerobic growth a gas phase of high-purity nitrogen (<0.001 % O_2) was used, and air was introduced at gradually increased rates for each successive aerobic sample, until fully aerobic conditions were reached. For this last aerobic sample the sparging rate was increased from 1 l air/min to 3 l air/min.

Both nitrogen and air were supplied from cylinders (Air Products). The gas flow rates were regulated by passage through Flostats and measured by Rotameter flow meters (G. A. Platon, Croydon, Surrey).

The culture was allowed to equilibrate at each new level of aeration for 18 h. The cells were then collected during a 6 h period into a chilled receiver at 2° and

harvested and fractionated as previously described[7]. A HUGHES'[31] press was used to break the cells.

Measurement of physical parameters

Oxygen. Mackereth type oxygen electrodes were used to measure oxygen concentrations of the inflowing and of the outflowing gas mixture, and a steam-sterilisable Mackereth electrode modified by BROOKMAN[33] was used to determine dissolved oxygen in the culture.

pH. The culture pH was controlled to ± 0.1 pH unit using automatic pH control coupled to a Model 23 A pH meter (Electronic Instruments., Richmond, Surrey).

E_h. The oxidation–reduction potential of the culture was measured with a Vibron electrometer (Electronic Instruments). The electrodes used were a standard calomel reference electrode and a platinum wire electrode of 5 cm length in the form of a spiral. The electrode system was tested for accurate read-out both before and after each experiment by immersion in a standard poised solution of ferricyanide – ferrocyanide and found to be correct to ± 10 mV.

Temperature. The temperature was controlled to ± 0.2° with a Kent temperature control unit (George Kent, London).

Growth yield

Yields for each growth condition were determined from the culture absorbance at 550 nm using the SP. 600 spectrophotometer (Unicam Instruments, Cambridge). Organism dry weight was then calculated using a calibration curve. The same absorbance to dry weight calibration was found to hold true for aerobically and anaerobically grown cells.

Enzyme assays

Aconitase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3), fumarase (L-malate hydro-lyase, EC 4.2.1.2), isocitrate dehydrogenase ($NADP^+$) (L_S-isocitrate: $NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) and membrane NADH oxidase were measured spectrophotometrically in a Cary-14 recording spectrophotometer. Hydrogenase (EC 1.12.1.1) was measured manometrically. Assays were carried out as described in previous publications[7].

Cytochrome measurement

Cytochromes were identified and measured with the Cary-14 spectrophotometer, using the method described in a previous publication[7].

ATP assay

ATP was measured using the luciferase assay described by COLE *et al.*[34].

RESULTS

The relationship between dissolved oxygen partial pressure and redox potential

Oxygen was measured using a membrane electrode similar to that described by MACKERETH, but modified by BROOKMAN[32] (see METHODS). These electrodes

measure the partial pressure of dissolved oxygen. In a number of continuous culture experiments oxygen partial pressure and redox potential were both measured as oxygen in the gas phase was varied. The results are shown in Fig. 1. The earliest significant changes appear in E_h which rises steeply: as this reaches the point of inflection the oxygen partial pressure increases. These figures are comparable for *K. aerogenes* and for *E. coli*, grown on either a complex medium or a glucose salts medium. The data for *E. coli* have been plotted on a logarithmic scale to emphasize the changes that occur at low aeration rates (Fig. 2). It seemed likely that oxygen was only easily measurable when it was present in quantities greater than that required to saturate demand by the cell: we therefore decided to relate changes in cell physiology to the redox potential.

Growth yield and redox potential

Growth yield was measured turbidimetrically and rises as aeration increases to a maximum figure above which yield was significantly reduced (Fig. 3).

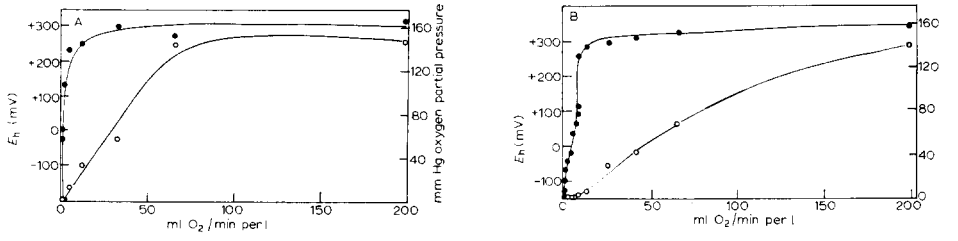


Fig. 1. The relationship between oxygen partial pressure and E_h in glucose limited chemostat cultures of *E. coli* K_{12} (A) and *K. aerogenes* (B). ●—●, E_h; ○—○, pO_2.

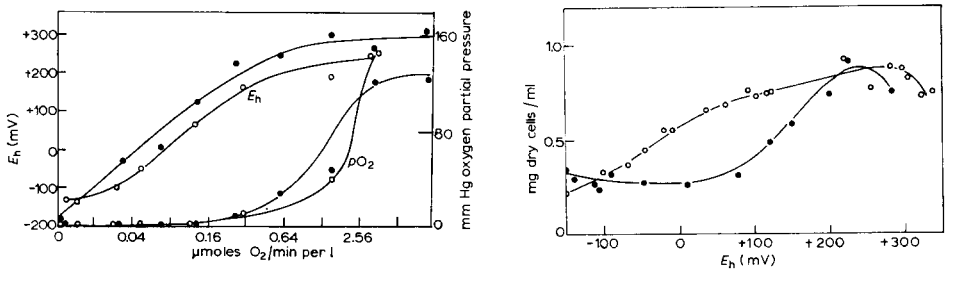


Fig. 2. The relationship between oxygen partial pressure and E_h in chemostat cultures of *E. coli* K_{12}. ●—●, glucose–salts medium; ○—○ glucose–tryptone–salts medium.

Fig. 3. Cell yield and E_h in chemostat cultures of: ●—●, *E. coli* (glucose–tryptone–salts medium); ○—○ *K. aerogenes* (glucose–salts medium).

Cytochrome levels and E_h

Facultative anaerobes such as *K. aerogenes* and *E. coli* have cytochromes a_1, a_2, b_1 and *o* when grown aerobically: they also form a low potential soluble *c*-type cytochrome under certain conditions when grown anaerobically. We relate cytochrome level to E_h in *E. coli* (Fig. 4) and in *K. aerogenes* (Fig. 5). Both organisms show maximum production of cytochromes a_2 and b_1 at around + 100 mV. At this E_h oxygen supply is probably limiting as it is barely detectable using the Mackereth electrode.

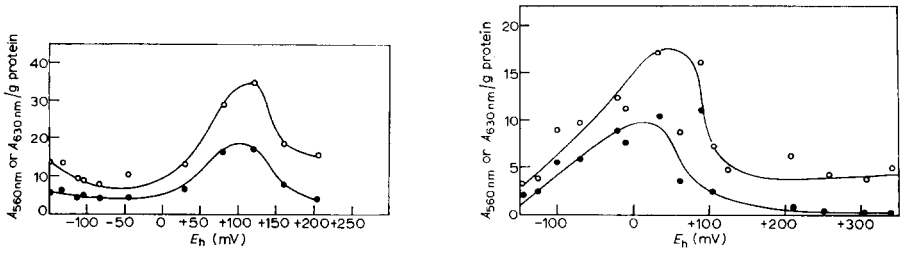


Fig. 4. Cytochrome levels and E_h in glucose–tryptone–salts-grown chemostat cultures of *E. coli* K_{12}. ○—○, cytochrome b_1; ●—●, cytochrome a_2.

Fig. 5. Cytochrome levels and E_h in glucose–salts-grown chemostat cultures of *K. aerogenes*. ○—○, cytochrome b_1; ●—●, cytochrome a_2.

Tricarboxylic acid cycle enzyme levels and E_h

Enteric bacteria have a functional tricarboxylic acid cycle whose constituent enzymes vary in activity depending on cultural conditions. Oxygen is the most significant regulatory effector in *E. coli*[12] and *K. aerogenes*[13]. The levels of representative tricarboxylic acid cycle enzymes are plotted as a function of E_h in both organisms (Figs. 6 and 7). There is an optimum E_h for synthesis of these enzymes as for cytochrome pigment production: however, this optimum is consistently higher and occurs at +200 to +300 mV. At this point free oxygen appears in measurable quantities in the culture and it is concluded that oxygen supply now exceeds demand.

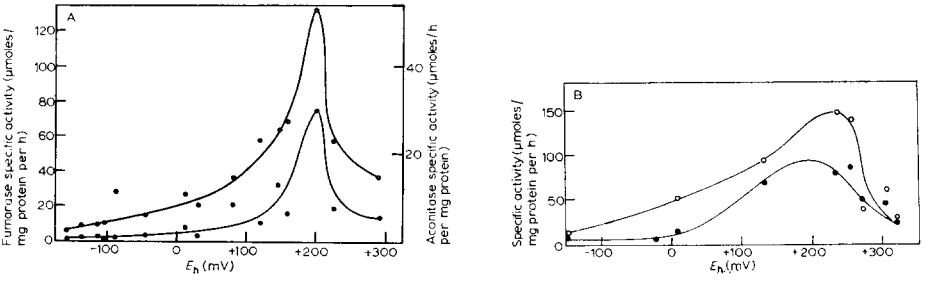


Fig. 6. A. Tricarboxylic acid cycle enzymes and E_h in glucose–tryptone–salts-grown chemostat cultures of *E. coli* K_{12}. ●—●, aconitase; ○—○, fumarase. B. Tricarboxylic acid cycle enzymes and E_h in glucose–salts grown chemostat cultures of *E. coli* K_{12}. ●—●, isocitrate dehydrogenase; ○—○, fumarase.

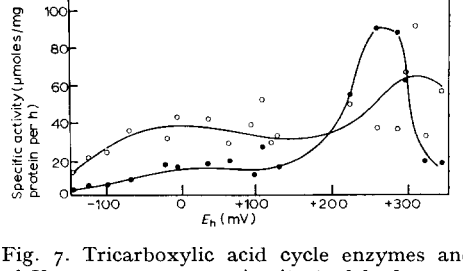
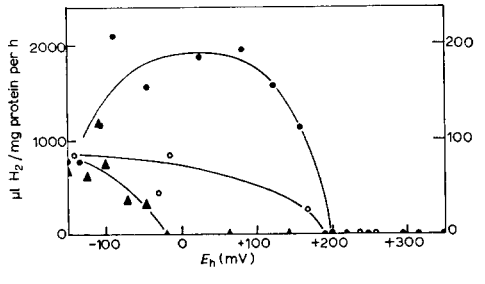


Fig. 7. Tricarboxylic acid cycle enzymes and E_h in glucose–salts-grown chemostat cultures of *K. aerogenes*. ●—●, isocitrate dehydrogenase; ○—○, fumarase.

There appears to be some species difference as the potential for maximum tricarboxylic acid cycle enzyme production is a little higher in *K. aerogenes* than in *E. coli*.

Hydrogenase activity and E_h

The anaerobic enzyme hydrogenase is repressed aerobically. This enzyme disappears as oxygen is increased in the medium. In all cases the enzyme is absent above about +200 mV in *E. coli* (Fig. 8). The strain of *K. aerogenes* used in this experiment had only low hydrogenase levels which disappeared at about +200 mV.



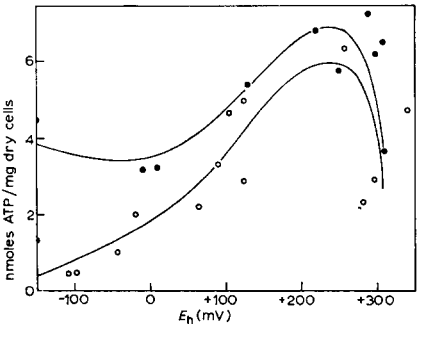


Fig. 8. Hydrogenase activity and E_h in chemostat cultures of: ●—●, *E. coli* K_{12} glucose–tryptone–salts medium; ○—○, *E. coli* K_{12} glucose–salts medium; ▲—▲, *K. aerogenes* glucose–salts medium. Left ordinate: ○—○ and ●—●; right ordinate: ▲—▲.

Fig. 9. Steady-state ATP pool level and E_h in glucose–salts grown chemostat cultures of: ●—●, *E. coli*; ○—○, *K. aerogenes*.

Steady-state ATP pool level and E_h

The steady-state level of ATP in both *E. coli* and *K. aerogenes* has been measured and related to culture E_h. (Fig. 9). The maximum for both organisms corresponds to the E_h at which cell yield and levels of tricarboxylic acid cycle enzymes are highest.

DISCUSSION

The transition to aerobiosis in Enteric bacteria.

These results suggest four separate physiological phases which can be identified during the transition from anaerobiosis to aerobiosis in both *E. coli* and *K. aerogenes*. These are:

Phase 1. Anaerobiosis, at an E_h of less than 0 mV: low levels of tricarboxylic acid cycle enzymes, cytochromes, steady-state ATP pool and growth yield; high levels of hydrogenase.

Phase 2. At around +100 mV: cytochrome synthesis is maximal. Steady state ATP pool, growth yield and tricarboxylic acid cycle enzyme levels are slowly increasing. Hydrogenase greatly reduced. Oxygen barely detectable.

Phase 3. Between +200 and +300 mV. Cytochrome levels low. Hydrogenase absent. Tricarboxylic acid cycle enzymes, steady-state ATP pool and growth yield maximal. Oxygen easily measured.

Phase 4. Above this level in carbon limited chemostat experiments all measured activities were reduced.

We might tentatively interpret these data as follows: in Phase 1 the cells are truly anaerobic, in Phase 2 with limiting oxygen available the terminal portion of the electron transport pathway to oxygen proliferates and is perhaps sterically and quantitatively altered such that the affinity for oxygen is increased. During this phase sufficient reductant is available from anaerobic metabolism without increasing levels of tricarboxylic acid cycle enzymes. In Phase 3 the terminal respiratory pathway reverts to a lower affinity for oxygen when the latter is present in excess. Reductant supply is increased by inducing enzymes of the tricarboxylic acid cycle. The cell is now operating most effectively energetically. This is reflected by a high steady-state ATP pool and growth yield. In Phase 4, excessive oxygen has some toxic effects or alternatively competes for reductant which could otherwise be required for biosynthesis.

These explanations have not yet been convincingly proven, but suggest lines for further experimentation. Our results have much in common with isolated work of others in this field. WHITE showed that poor aeration led to increased synthesis of cytochromes, respiratory dehydrogenase enzymes and quinones in *Hemophilus parainfluenzae*, he has demonstrated an increased affinity for oxygen when his cells are grown under these conditions[35]. It seemed that the developing respiratory components were not coordinately regulated and work of the type we have described might show different phases during adaptation of *H. parainfluenzae* to increasing oxygen.

The use of redox potential

The redox potential of a bacterial culture is a complex parameter not at present clearly understood. It measures a variety of soluble oxidised and reduced couples capable of reacting at the surface of a gold or platinum electrode. Such interesting couples may be substrates or metabolic products of microbial growth. They may be other medium constituents that do not directly affect microbial growth or they may be minor cellular components that have leaked into the medium. The redox potential is thus a numerical estimate of the oxidising or reducing nature of the environment. It seems possible that certain physiological changes in facultative anaerobes can be related to redox potential. The question arises: is E_h merely a useful number that changes dramatically as aeration increases and when oxygen can not easily be measured with oxygen-specific electrodes, or is it a parameter that has some deeper significance in cell regulatory mechanisms?

The answers to these questions are not immediately clear. A lot depends on the nature of the important redox couples that the electrode "sees". For instance we believe that the reduction of oxygen in the absence of cells is not an important couple and is swamped by other redox reactions in a microbial culture. An indication of this might be that only a small change in E_h is observed when the partial pressure of oxygen rises sharply in our continuous culture experiments (Fig. 1). The potential we measure seems to be a cell-orientated parameter and there might thus be an important connection between the culture E_h and the physiological make up of the cell. Two possibilities exist: that the external E_h is seen by the cell and appropriate regulatory mechanisms came into play or, much more likely, that the external E_h is a reflection outside the cell of events occurring in it. It would be satisfying if one of these explanations were completely true, but the whole situation is made

more complex by the possibility of redox couples which interact with the electrode but not with the cell. Preliminary studies with some other electron acceptors show that nitrate and possibly nitrite as well as oxygen do not significantly alter the E_h of a culture other than through the cell. These results will be reported elsewhere.

The importance of redox potential has been stressed by a number of workers. As early as 1930 KNIGHT AND FILDES[36] showed that spores of anaerobic bacteria could not germinate above a very consistent E_h figure, more recently DANIELS *et al.*[28] indicates that tissue culture cells cannot initiate growth if the E_h is high. DANIELS in a personal communication has indicated that this may also be true for bacterial cultures. It becomes increasingly clear that E_h may be one of the most fundamental, though perhaps the most complex, indicators of the physiological state of microbial cultures.

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