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A STUDY OF THE EFFECT OF GROWTH CONDITIONS ON CHEMOSTAT-GROWN *KLEBSIELLA AEROGENES* AND KINETIC CHANGES OF A 500-nm ABSORPTION BAND

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SUMMARY

Measurement of the cytochrome content of cells grown under different aeration conditions showed that cytochrome *o* did not go through the same adaptive changes as did cytochromes a_1 and a_2, and no correlation was found between cytochrome content of cells and their potential respiration rate. Cells grown at low growth rate demonstrated increased content of cytochrome a_2.

A peak of absorption at 500 nm was observed in cells grown at 0.04 h^{-1} and in samples incompletely oxidised. Kinetic studies using a dual-wavelength spectrophotometer showed two types of absorption at 500 nm. One type appeared on aerating anaerobic cells and disappeared slowly with time, and the other appeared transiently as the cells became anaerobic.

INTRODUCTION

Continuous cultures of *Klebsiella aerogenes* have been found to respire at a higher rate at low oxygen tensions (< 2 mm Hg) than at higher oxygen tensions[1], and sustained oscillations of respiration rate may be obtained in the transition phase between excess and limited oxygen states. A model for such oscillations has been proposed[2] that is analogous to an electrode system with a region of negative slope in the current/potential curve. Although the Q_{O_2} of the cells may increase by 20 % when the oxygen tension is lowered to below 2 mm Hg, Harrison and Maitra[3] found that there is no accompanying rise in ATP content and suggested that this may be explained by a fall in P/O ratio caused by an alternative path for electrons becoming operational. In order to investigate this phenomenon further, studies were carried out on the cytochrome system of *K. aerogenes* grown at various oxygen tensions.

Early work by Moss[4,5] showed that the cytochromes a_1 and a_2 of *K. aerogenes* and *Escherichia coli* were adaptive, being found in large amounts under partially aerobic conditions but in very small amounts at high oxygen tensions. Similar effects have been found in other species of bacteria[6]. However, the photometric techniques used by these workers require that cells be harvested and concentrated before the

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cytochromes can be measured and only detect those pigments which have a pronounced peak of absorption in the reduced form. The split-beam spectrophotometer technique developed by Chance[7] makes possible the detection and measurement of small quantities of cytochromes and the detection of pigments with absorption peaks in the oxidised form. This technique has been widely used to study the cytochromes of bacteria[8]. More recently, Wimpenny and Cole[9] have reported that when *E. coli* is grown with nitrate as electron acceptor under anaerobic conditions, the cytochromes demonstrate the same pattern of induction as they do with oxygen as electron acceptor.

By coupling the use of liquid-nitrogen temperatures with the split-beam recording spectrophotometer technique it is possible to study the cytochromes of low concentrations of cells which are fixed in the required steady state[10]. In this work, the split-beam spectrophotometer has been employed to study the effect of oxygen tension on the cytochrome content of chemostat cultures of *K. aerogenes* and an attempt has been made to study the steady state condition of the cytochromes. Also, the dual wavelength spectrophotometer[11] has been employed to follow the kinetics of changes in respiratory pigments of cells taken from the chemostat.

METHODS

The organism used was *K. aerogenes* (N.C.I.B. 8017). The medium was that of Harrison and Pirt[1]. A 1-l magnetically stirred chemostat vessel was used to grow the organisms. The temperature of the vessel was maintained at 30 $\pm$ 0.05 °C, and the pH controlled at 6.0 $\pm$ 0.02 by the addition of 2 M NaOH. Aeration was by means of a baffled vortex and the oxygen tension in the vessel was altered by changing the partial pressure of oxygen in the gas supply. Dissolved oxygen tension was recorded by means of a Mackereth type electrode (Mackereth[12]) supplied by E.I.L. Company Limited, Richmond, Surrey. The culture was grown under glucose-limited conditions for all the experiments reported here.

The methods of taking samples for measurement in the split-beam spectrophotometer were as follows:

(1) Samples which were to be analysed immediately, were taken directly from the chemostat and either supplied with glucose and allowed to become anaerobic to reduce the cytochromes, or bubbled with oxygen for 10 min to fully oxidise the cytochromes.

(2) Some samples had to be stored for a few days before they could be analysed. Slow freezing was found to destroy most of the respiratory properties, and so the cells were frozen very rapidly. For this purpose, 100-ml polypropylene centrifuge tubes were stood in a beaker containing fibreglass to provide thermal insulation and the tubes were half-filled with liquid nitrogen. The sample port was opened and after allowing the first 5 ml to flow to waste, a sample of about 20 ml was allowed to flow directly into the liquid nitrogen, effecting rapid freezing of the sample. The sample was then stored in a liquid nitrogen Dewar flask until required. For analysis, the sample was thawed and used as for a fresh sample. On thawing these samples retained the respiration rates of the fresh cells.

Difference spectra were obtained using a split-beam recording spectrophotometer (made at the Johnson Foundation, University of Pennsylvania). The technique has been described in full[13,14]. Kinetic studies of cytochromes were carried out using the dual wavelength spectrophotometer technique developed by Chance[15]. The instrument used was built at the Johnson Foundation.

Cytochrome *o* was estimated from the difference spectra of CO-treated reduced cells using reduced cells as reference. Estimations of cytochromes b_1 and a_2 were obtained from difference spectra of reduced *minus* oxidised cells.

RESULTS

Cytochrome content

A typical reduced *minus* oxidised difference spectra of *K. aerogenes* is shown in Fig. 1. The peaks at room temperature are at 630, 595, 560, 535, 435 and 430 nm, indicating the presence of cytochromes a_1, a_2 and b_1 (ref. 16), but at liquid-nitrogen

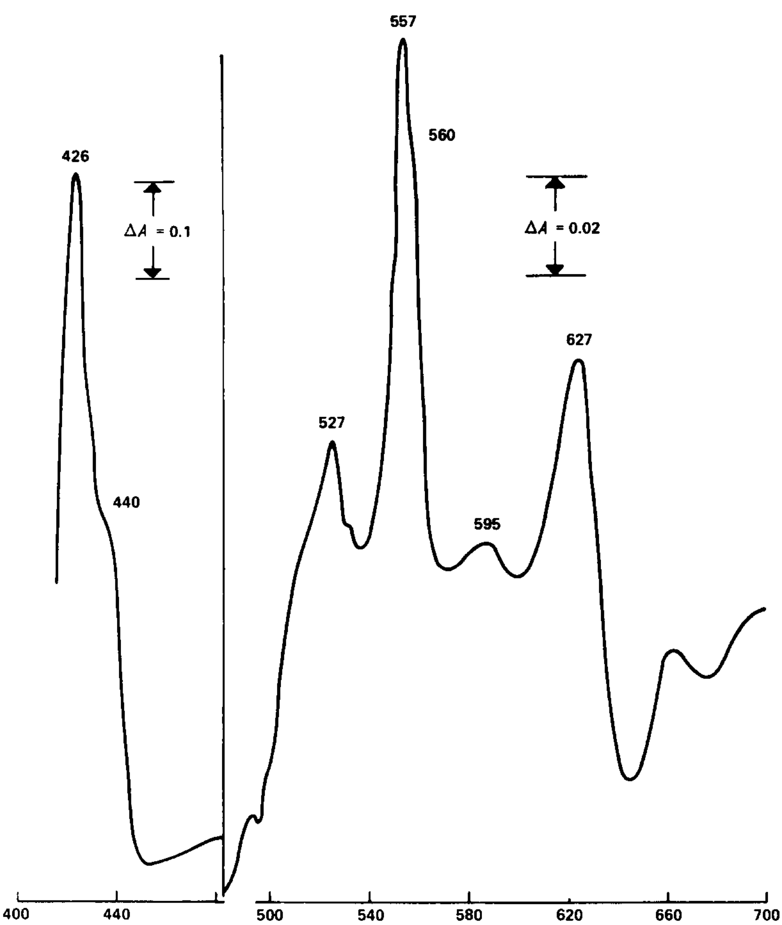

Fig. 1. Reduced *minus* oxidised difference spectrum of cells of *K. aerogenes* grown under limited-oxygen conditions (dissolved oxygen tension <0.5 mm Hg). Growth rate = 0.2 h^{-1}. Cell concentration was 22 g/l. The spectrum was obtained at liquid-nitrogen temperature.

TABLE I

CHANGES IN THE CYTOCHROME CONTENT OF *K. aerogenes* GROWN UNDER DIFFERENT OXYGEN TENSIONS

Absorption of cytochromes was measured in intact cells at liquid-nitrogen temperature by means of a split-beam spectrophotometer. Cytochromes *a* and *b* were estimated from reduced *minus* oxidised difference spectra and cytochrome *o* from CO-treated reduced *minus* untreated reduced difference spectra.

% O_2 in gas supply	*Dissolved O_2 tension (mm Hg)*	*Cytochrome content ($\Delta A \times 10^3$/mg dry wt)*		
		b_1 (560 nm)	*a_2 (630 nm)*	*o (417 nm)*
100	420	2.4	<0.01	1.2
50	220	2.1	<0.01	2.5
21	57	2.5	<0.01	2.2
15	5.3	2.1	<0.01	2.9
9	<0.5–2.5 *	3.1	1.24	2.2
4	<0.4 **	2.7	2.17	1.9
0	0	2.6	1.24	1.9

* Oscillations in O_2 tension and respiration rate occurred at this oxygen feed.
** The dissolved oxygen tension was below the lowest sensitivity of the measuring electrode.

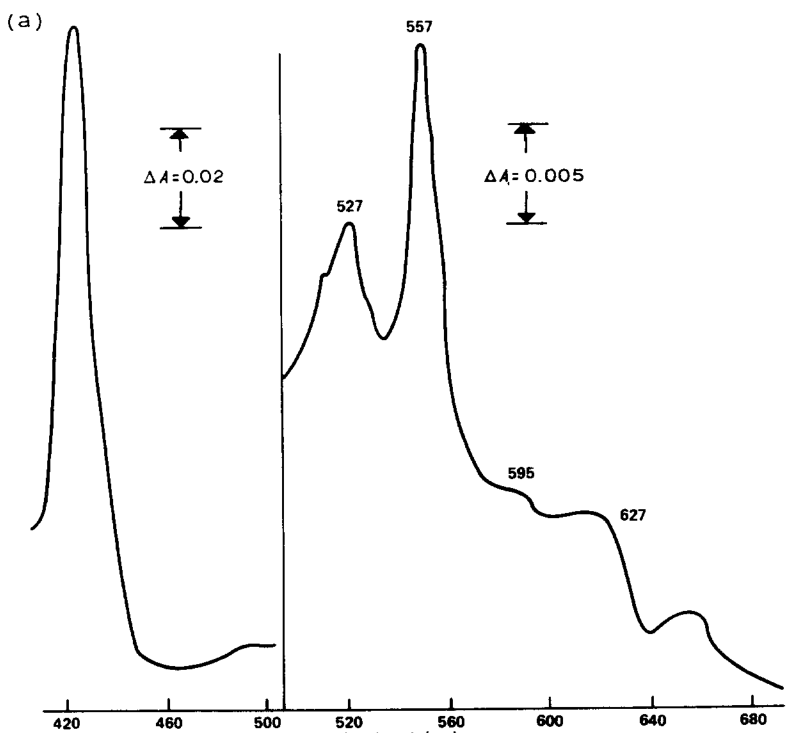

Fig. 2a. Reduced *minus* oxidised difference spectrum of cells of *K. aerogenes* grown under fully aerobic conditions (dissolved oxygen tension > 10 mm Hg) at a growth rate of 0.2 h^{-1}. Cell concentration was 10 g/l. The spectrum was obtained at liquid-nitrogen temperature.

temperature these are displaced about 3 nm towards the ultraviolet. Table I shows the change in spectrum with changes in the oxygen tension at which the organisms were grown. In agreement with the work of Moss[5], the amount of cytochrome a_2 was found to be extremely low until the oxygen tension was lowered to below 2.5 mm Hg, whereas cytochrome b_1 content was fairly constant. From Table I it is quite clear that the cytochrome *o* concentration changes much less with oxygen tension than does that of cytochrome a_2. Accurate values for the molar extinction coefficients of these cytochromes are not available and so it is not possible to compare the quantities of cytochromes directly. The data given in Table I indicate only relative changes in the amounts of these cytochromes.

The effect of growth rate on absorption spectrum

Reduced *minus* oxidised difference spectra were obtained from cells grown at different growth rates to determine whether factors other than oxygen tension could influence cytochrome formation. Figs 2a and 2b show a comparison between the spec-

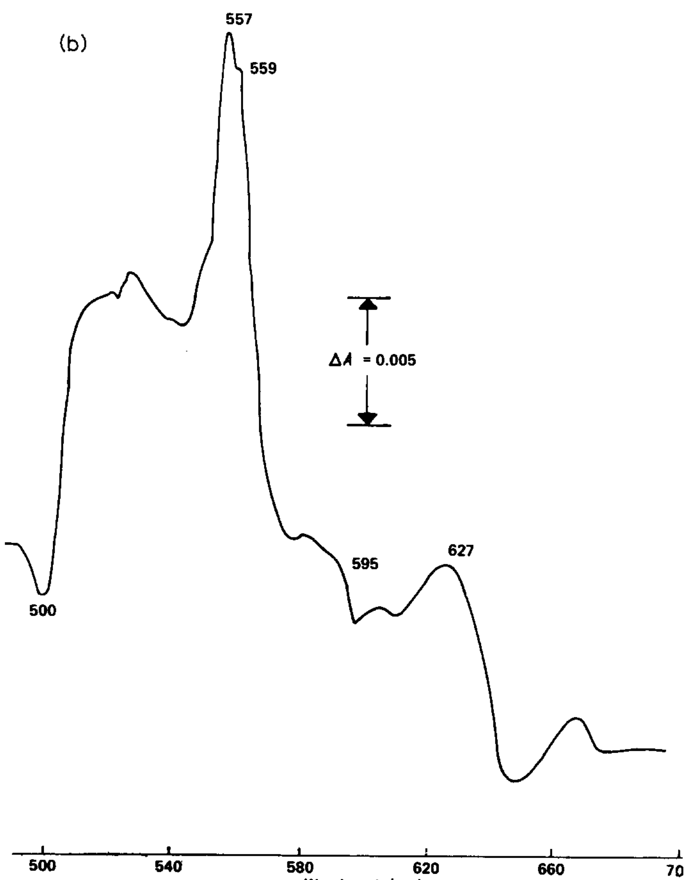

Fig. 2b. Reduced *minus* oxidised difference spectrum of cells of *K. aerogenes* grown under fully aerobic conditions (dissolved oxygen tension > 10 mm Hg) at a growth rate of 0.04 h^{-1}. Cell concentration was 10 g/l. The spectrum was obtained at liquid-nitrogen temperature.

trum of cells grown at a moderate (0.2 h^{-1}) growth rate with those of cells grown at a very low growth rate (0.04 h^{-1}). From this it can be seen that significant quantities of cytochromes a_1 and a_2 were formed under conditions of low growth rates even though oxygen was in excess. A trough, indicating absorption in the oxidised cells, appeared at 500 nm in the cells grown at 0.04 h^{-1} but was not present in the fully-reduced *minus* fully-oxidised spectrum of cells grown at higher growth rates.

The main peak of the cytochrome b_1, which is at 557 nm at liquid-nitrogen temperature, displays a shoulder at about 559 nm (Fig. 1). In the cells grown at a low growth rate, this shoulder was much more pronounced and was almost as large as the 557-nm peak (Fig. 2b). Possibly, this represents a separate unidentified pigment rather than a splitting of the b_1 peak.

Absorption at 500 nm

A peak of absorption had been observed at 500 nm in oxidised cells grown at 0.04 h^{-1}. A trough at 500 nm was observed on occasions in samples in which the cytochromes were incompletely oxidised, but disappeared on prolonged aeration of the sample. For this reason it was decided to follow changes in the spectrum of a sample on changing during the transition from the oxidised to the reduced condition by repeated scanning with a split-beam spectrophotometer. To this end, the reference

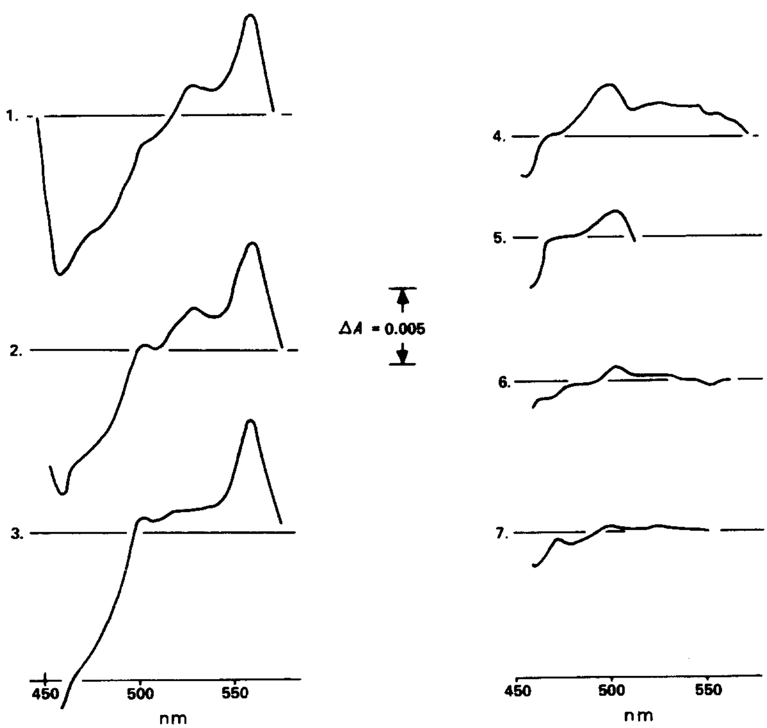

Fig. 3. Difference spectra obtained from cells during an aerobic to anaerobic transition. The reference cuvette was filled with anaerobic cells supplied with glucose, and the measuring cuvette was filled with cells bubbled with air for 5 min; the spectrum was scanned at approximately 30-s intervals while the cells in the measurement cuvette became anaerobic. The spectra were obtained at room temperature. The cells were from a culture growing in the limited-oxygen state.

cuvette was filled with a sample that was allowed to go anaerobic after the addition of glucose while in the other cuvette, a sample was bubbled with air for 5 min. Immediately on ceasing the bubbling, the difference spectrum was scanned at 30-s intervals. The resulting spectra are shown in Fig. 3. From this, it can be seen that as the cytochrome b_1 peak disappeared, a peak appeared at 500 nm which then disappeared again as the sample became anaerobic. The cells used in this experiment were harvested from a fully aerobic culture so the amounts of cytochromes a_1 and a_2 present were very small.

In order to obtain a more accurate assessment of the kinetics of the system, a dual-wavelength spectrophotometer was used. A sample from a culture growing under limited-oxygen conditions was oxidised by bubbling with air for 5 min and the change in cytochromes was observed as the sample became anaerobic using the wavelength pairs 560/510 nm for cytochrome b_1, 450/510 nm for flavoprotein and 500/510 nm. The latter pair was chosen because, although 510 nm was not a true isobestic point, there was no inflection between these wavelengths so that the changes in relative absorption would always be an indication of a change in absorption at 500 nm.

The results are shown in Fig. 4. In these tracings, it cannot be assumed that the time taken for the sample to become anaerobic will be the same in each case. The tracings for cytochrome b_1 and flavoprotein are as would be expected, the pigment

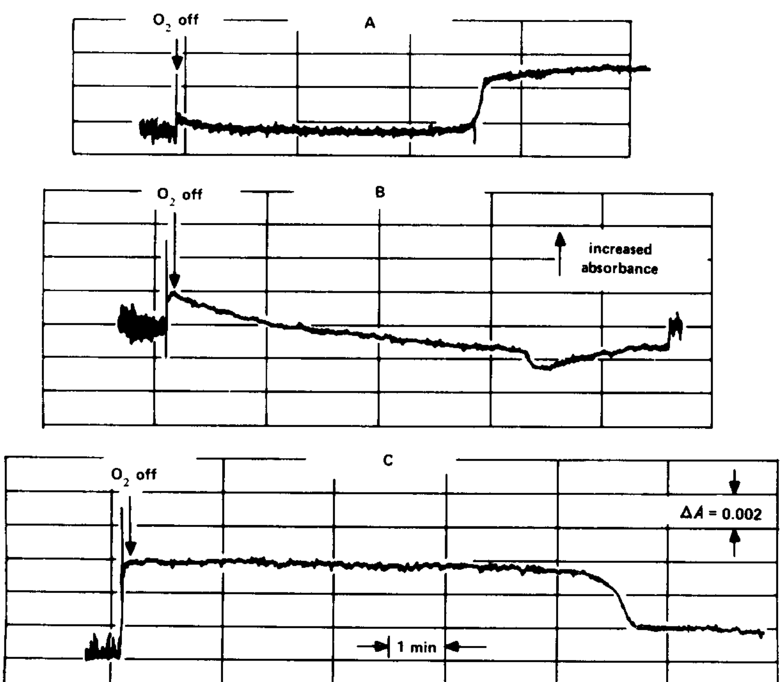

Fig. 4. Tracings obtained, using the dual-beam spectrophotometer from cells harvested from a culture in the limited-oxygen state after bubbling the sample for 5 min with air. (A) Cytochrome b_1 absorption (560–510 nm). (B) P-500 absorption (510–500 nm). (C) Flavoprotein absorption (550–510 nm).

changing its form rapidly as soon as the oxygen is exhausted. However, the 500/510 nm pair is quite extraordinary in that there is an initial slow decrease in absorption which reaches a steady state apparently before oxygen is exhausted and then, presumably at the onset of anaerobiosis, there is a rapid decrease in absorption followed by a slow recovery to the steady state. The slow initial decrease in absorption seemed to be only time dependent and the rapid change appeared to occur only when the sample became anaerobic. This is demonstrated in Fig. 5 where air was bubbled through the sample for less than 1 min so that the oxygen would be exhausted more rapidly. Here it can be seen that the rapid fall in absorption, which is probably associated with anaerobiosis, occurs before the slow time-dependent fall is completed.

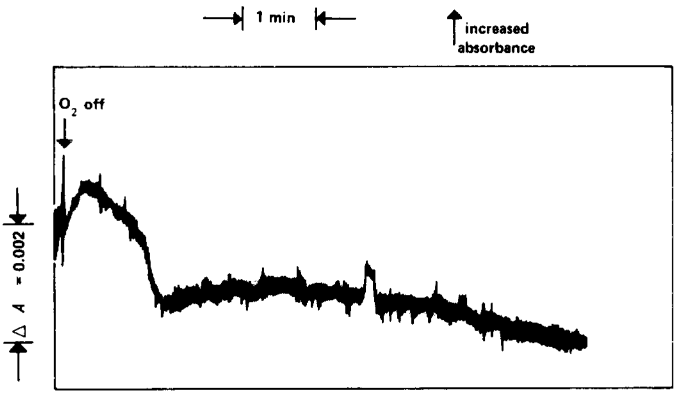

Fig. 5. Tracing of P-500 obtained, using the dual-beam spectrophotometer, from cells harvested from a culture in the limited-oxygen state, after bubbling the sample with air for 5 min (500–510 nm).

The results obtained with the dual-wavelength spectrophotometer agree with the observations from rapid scanning data obtained using the split-beam spectrophotometer.

DISCUSSION

That facultative anaerobic bacteria may increase their content of terminal oxidase at low oxygen tension has been known for many years[4] but the reason for this adaptation remains obscure. Cytochrome *o* is generally regarded as a terminal oxidase[18], but the purpose of a second terminal oxidase in the cell is not known. The fact that cytochrome *o* is not adaptive in the same way as cytochrome a_2 would indicate that it has a somewhat different, if overlapping, role.

There is no correlation between the quantity of cytochromes in the cell and the 'potential' respiration rate (*i.e.* the maximum respiration rate demonstrated by the cells in the presence of excess oxygen and substrate). The highest potential respiration rates found in *K. aerogenes* occurred in anaerobic cells[17] where the cytochrome a_1 and a_2 content is low. Possibly an increased content of cytochrome a_2 under oxygen-limited conditions may prevent the terminal oxidase reaction rate from becoming limiting for respiration at oxygen concentrations approaching the K_m for the reaction. However, high contents of cytochrome a_1 and a_2 were found in slow growing of

K. aerogenes although oxygen was in excess. Under these conditions the Q_{O_2} of the cells is lower than in faster-growing cultures[17], although the latter have very low contents of cytochromes a_1 and a_2. Therefore, it seems likely that the changes in cytochrome content have a function unrelated to control of respiration rate or may be a chance result of metabolic disturbance.

Other workers have detected pigments absorbing in the 500-nm region in bacteria[19]. Lindenmayer and Smith[20] studied a pigment in yeast with an absorption band at 503 nm. A comparison of the yeast 503-nm pigment with the time-dependent 500-nm absorption found in *K. aerogenes* is shown in Table II. They differ in that the 503-nm pigment of yeast absorbs in anaerobic cells while the 500-nm pigment studied here absorbs in aerated cells. Otherwise, the pigments show similarities in that the change from the aerobic to anaerobic state is slow and the peak disappears on prolonged aeration or prolonged anaerobiosis. It is possible that these are related compounds which are sensitive in different degrees to the redox potential inside the cell. Lindenmayer and Smith[20] suggested that the 503-nm pigment might be a semiquinone form of ubiquinone but they considered that the pigment was involved in reactions not on the main electron transport pathway. Itoh and Nosoh[21] came to similar conclusions. Other workers[22] have postulated that the pigment is protoporphomethenic.

TABLE II

A COMPARISON OF THE TIME-DEPENDENT 500-nm PIGMENT WITH THE 503-nm PIGMENT OF YEAST

Time-dependent P-500	*Yeast P-503 (ref. 20)*
Absorbs in freshly-aerated cells	No absorption in aerated cells
Bleaches slowly with time	Absorption increases slowly on anaerobiosis
Absent in well-aerated cells	Disappears on aeration in absence of glucose
Absent in anaerobic cells	Disappears when cells are left anaerobic (ref. 21)
No absorption in dithionite-treated cells	Bleached by dithionate

Possibly, the time-dependent changes observed at 500 nm in this work are caused by a compound, absorbing at 500 nm which is formed when anaerobic cells are aerated and then dissociates in a time-dependent manner, independently of oxygen tension. The transient changes obtained at 500 nm as the cells become anaerobic appear to be caused by a totally different mechanism to the time-dependent changes in that they can occur separately and are more rapid. This substance is peculiar in that it appears to be bleached only at one stage while the cells are on the verge of becoming anaerobic, and has the same absorption in the aerobic and anaerobic forms. This is difficult to explain in terms of known processes of the respiratory chain, but may represent reactions which occur only at low oxygen tensions and could possibly be involved in the mechanism for increasing respiration rates under oxygen-limited conditions[1].

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