A Re-examination of the Cytochromes of *Escherichia coli* using Fourth-order Finite Difference Analysis: Their Characterization under Different Growth Conditions and Accumulation during the Cell Cycle

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The cytochromes of *Escherichia coli* K12 have been studied using low-temperature (77 K) difference spectrophotometry. Numerical (i.e. fourth-order finite difference) analysis resolved the α band of reduced *minus* oxidized spectra of whole cells from aerobically grown cultures into five components, with absorption maxima at 548, 551.5, 555.5, 560 and 563 nm. Using the same differencing intervals, numerical analysis of cells grown under oxygen-limited conditions revealed only two components, at 555.5 and 559 nm. Similar analysis of cells grown anaerobically with fumarate as electron acceptor revealed four absorption maxima, at 548, 550.5, 555.5 and 559 nm. Membrane particles from aerobically grown cells showed the same absorption bands as intact cells; preliminary evidence was obtained for a component of the 555.5 nm band that could be relatively easily washed from the membrane. The contribution of cytochrome *o* or its CO-liganded form to the α region could not be determined by numerical analysis. We conclude, in contrast to a previous application of numerical analysis to the cytochromes of *E. coli*, that growth under anaerobic or oxygen-limited conditions results in the appearance of cytochromes spectrally distinct from those in aerobically grown cells. An attempt has been made to reconcile the presence of multiple components detected in the α region by numerical analysis of aerobically grown cells with the diverse components described by others.

Quantification of cytochromes revealed by numerical analysis in aerobically grown cells separated into size (and thus age) classes by zonal centrifugation showed that the major components accumulated continuously, probably exponentially, throughout the cell cycle.

INTRODUCTION

Escherichia coli is a facultative aerobe whose respiratory chain composition varies with the availability of oxygen or the replacement of oxygen by alternative electron acceptors (Haddock & Jones, 1977). Despite intensive study over the last ten years, the identity and sequence of the cytochrome components of the respiratory chain are not fully understood. A widely accepted, but probably simplistic, view (Haddock, 1980) is that the quinol-oxidizing ('high potential') segment of the respiratory chain comprises cytochromes b_{556} and *o* in aerobically grown cells. Under conditions of low oxygen tension (Rice & Hemphling, 1978), or in the presence of inhibitors of cytochrome *o* function, such as low concentrations of cyanide (Ashcroft & Haddock, 1975), or under sulphate-limited conditions (Poole & Haddock, 1975), concomitant synthesis of cytochromes b_{558} and *d* occurs. The *b*-type cytochromes formed under various conditions are potentiometrically, spectrally and kinetically distinct (Reid & Ingledew, 1979; Haddock *et al.*, 1976; Poole & Chance, 1981).

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This view is in marked contrast to that proposed by Shipp (1972) who studied the α band (previously attributed to 'cytochrome b_1 ') of spectra of *E. coli* using fourth-order finite difference analysis. He concluded that absorption bands of five or more different pigments contributed to the α band, and tentatively assigned them to two *c*-type and three *b*-type cytochromes. More importantly, the same set of resolved peaks were detected under all growth conditions examined, which included anaerobiosis.

In an attempt to resolve this paradox we have re-examined, using fourth-order finite difference analysis, the composition of the α band in difference spectra of *E. coli* grown under various conditions, and have found much greater differences in the position and intensity of the constituent bands than reported by Shipp. In addition, the ability to resolve the composite α band has enabled us to quantify the major components during the cell cycle. Parts of this work have been presented previously (Scott & Poole, 1980).

METHODS

Organism and growth conditions. *Escherichia coli* K12 (strain A1002) was grown aerobically or under oxygen-limited conditions in a medium containing 40 mM-succinate as carbon source supplemented with casein hydrolysate as described by Poole *et al.* (1979). The maintenance of stock cultures, the growth of starter cultures and the large-scale aerobic cultivation of cells have been described previously (Scott *et al.*, 1980). For the cultivation of cells under oxygen-limited conditions, the speed of the stirrer in the fermenter was reduced from 1100 rev. min⁻¹ to 300 rev. min⁻¹. Under these conditions the oxygen transfer rate (Scott *et al.*, 1980) was reduced from 54.2 mmol O₂ l⁻¹ h⁻¹ (for aerobically grown cells) to 0.3 mmol O₂ l⁻¹ h⁻¹. The oxygen tension in the growth vessel was continuously monitored using an ethylene oxide-sterilized Mackereth electrode (silver cathode, lead anode) inserted into the culture.

For growth under strictly anaerobic conditions, the same medium was used except that the succinate was replaced by glycerol (0.5%, v/v) and 50 mM-sodium fumarate was added as terminal electron acceptor. The medium was supplemented with 1 μ M-K₂SeO₃ and 1 μ M-ammonium molybdate (Haddock *et al.*, 1976). Starter cultures (40 ml in 100 ml Erlenmeyer flasks) were grown for 20 h at 37 °C under reduced pressure in an evacuated desiccator. Large-scale anaerobic growth of cells was carried out in the fermenter vessel described above. After autoclaving and during cooling, the medium (4 l) was sparged slowly with N₂. It was then inoculated with 40 ml of starter culture and N₂ gas (99.9% minimum purity) was passed into the headspace of the fermenter at 120 ml min⁻¹ to maintain anaerobiosis.

Harvesting of cells. Cells grown under aerobic conditions were harvested towards the end of exponential growth when the apparent absorbance (A_{600}), measured in cuvettes of 1 cm path length in a Pye Unicam SP1700 spectrophotometer, was about 0.7. Cells grown under oxygen limitation were harvested 24 h after inoculation (A_{600} about 0.2). Anaerobically grown cells were harvested at the end of exponential growth, when A_{600} was about 0.4. All cells were harvested using a continuous action rotor (Scott *et al.*, 1980) and washed once with 50 mM-Tris/HCl, 2 mM-MgCl₂, 1 mM-EGTA (pH 7.4).

Cell cycle analysis of aerobically grown cells. Growth of cells, harvesting and cell cycle fractionation into different size classes were exactly as described previously by Scott *et al.* (1980, 1981).

Subcellular fractionation of aerobically grown cells. Cells were harvested, washed as described above and then broken by ultrasonic disruption as described by Gibson *et al.* (1980) except that the end diameter of the sonicator probe was 9.5 mm. The whole sonicate was fractionated as described by Poole & Haddock (1974). The first pellet obtained by high-speed centrifugation was washed in 50 mM-Tris/HCl, 2 mM-MgCl₂, 1 mM-EGTA (pH 7.4), followed by a further wash with either the same buffer (to give 'membrane particles') or with 1 mM-Tris/HCl, 1 mM-MgCl₂ (pH 7.4) (to give 'depleted membrane particles'). The terms 'membrane particles' and 'depleted membrane particles' are used here in preference to earlier descriptions (Poole & Haddock, 1974) for reasons given previously (Poole *et al.*, 1980b).

Difference spectra and their numerical analysis. Difference spectra were obtained at 77 K using a Pye Unicam SP1700 spectrophotometer and low-temperature attachments as described previously (Salmon & Poole, 1980; Poole *et al.*, 1980a). Samples for cytochrome spectra were divided into two cuvettes. The reference sample was oxidized by adding 10 μ l H₂O₂ (20 vol.); after cessation of bubbling, the sample was frozen by plunging the cuvette into liquid nitrogen. The contents of the other (sample) cuvette were reduced with a few grains of Na₂S₂O₄ and frozen after 2 min. Where indicated, reduced samples were liganded with CO by bubbling a slow stream of CO through the suspension in a small tube for 2 min, before transfer to the cuvette and freezing. Numerical analyses of spectra were carried out using the algorithm of Butler & Hopkins (1970) as described by Salmon & Poole (1980). Wavelengths are quoted to the nearest 0.5 nm to indicate reproducibility but a precision of 1 nm is a more realistic

estimate of that obtainable with this instrumentation. The salient features of this type of analysis are: (1) it allows resolution of neighbouring absorption bands by effectively decreasing their band width; (2) when the differencing intervals chosen for the four orders of analysis are similar but not equal, signal-to-noise properties of the spectrum are improved; (3) the procedure discriminates strongly in favour of bands in the original spectrum that have small band widths – indeed, components with broad band widths may not be resolved at all. Numerical analysis of the effects of CO on absorption bands were performed on difference spectra of the CO-liganded, reduced samples *minus* oxidized samples rather than on conventional 'CO difference spectra' (i.e. CO + reduced *minus* reduced); the latter type of difference spectra exhibit distinct troughs that confound the fourth-order finite difference analyses.

Cell numbers and volumes. These were determined using a Coulter counter Z_{BI} and Channelyzer C1000 (Coulter Electronics, Luton L43 3RH, U.K.) as described by Scott *et al.* (1980).

Protein determinations. The total protein content of concentrated suspensions of cells was determined in 0.5 M-NaOH extracts as described by Herbert *et al.* (1971), with dry bovine plasma albumin as standard.

Measurement of oxygen uptake. Respiration rates of culture samples were measured with a Clark-type oxygen electrode at 37 °C as described by Poole (1977). Samples (2 ml) were transferred from the fermenter to the electrode vessel and aerated by vigorously stirring with the electrode vessel open to the atmosphere. Oxygen consumption rates in the closed vessel were then determined.

RESULTS

Growth of E. coli under different conditions

The growth characteristics of cells grown aerobically in the succinate-containing medium have been described earlier (Poole *et al.*, 1980*a*). Exponential growth was biphasic, giving a final yield of about 4×10^9 cells ml⁻¹.

Oxygen-limited growth of *E. coli* in batch culture has not been well defined previously. Characteristics of growth in the succinate-containing medium under these conditions are shown in Fig. 1. After an initial lag period of 1.5 h, cell numbers increased rapidly for a further 1.5 h; the growth rate of the culture then decreased. Under these conditions there was no period of exponential growth; the final yield was 1×10^9 cells ml⁻¹. The mean cell volume increased initially during the lag phase, remained constant during the period of rapid growth and then decreased during the remainder of the experiment. These volume changes are similar to those observed during batch growth of well aerated cultures (Poole, 1977). The dissolved oxygen tension of the medium fell rapidly from 100% saturation at inoculation to zero after 3 h. Exhaustion of dissolved oxygen was concomitant with the reduction in the rate of increase in cell numbers and the decrease in mean cell volume. The potential rate of oxygen uptake by the culture increased rapidly during the first 3 h after inoculation but increased only gradually after exhaustion of the dissolved oxygen to reach a final rate of about 20 nmol O₂ min⁻¹ ml⁻¹.

Under anaerobic conditions, cultures grew exponentially (doubling time 120 min) for about 5 h following inoculation, after a 30 min lag period. The doubling time then increased to 180 min and exponential growth continued for a further 7 h. The growth rate decreased as the culture entered the stationary phase. The final yield was about 3×10^9 cells ml⁻¹.

α absorption bands of aerobically grown cells

The α region of a dithionite-reduced *minus* peroxide-oxidized spectrum of intact cells from an aerobically grown culture is shown in Fig. 2(*a*). Small absorption bands in the α region due to cytochrome *a*₁ (590 nm) and cytochrome *d* (628 nm) were also visible (not shown). The only peak clearly resolved in the spectral region shown was at 556 nm but the shape of the spectrum suggested considerable heterogeneity, which was revealed by fourth-order finite difference analysis (Fig. 2*b*). At small differencing intervals (1.8, 1.4, 1.2 and 1.0 nm), peaks were observed at 548, 551.5, 555.5, 560 and 563 nm, in good agreement with the positions found by Shipp (1972) who used larger differencing intervals (4.8, 3.6, 3.0 and 2.4 nm). Analysis of the same spectrum (Fig. 2*a*) using the differencing intervals employed by Shipp

Fig. 1. Growth of *E. coli* in succinate-containing medium under conditions of oxygen limitation. The variables shown are cell numbers (●), mean cell volume (○) and oxygen uptake rate (□). The dissolved oxygen tension of the medium is shown as a continuous recording.

(1972) showed components at 546, 555 and 563 nm but not the minor components at 551.5 and 560 nm (results not shown). The important point is that bands at wavelengths equal, or similar, to those reported by Shipp (1972) were resolved in spectra of aerobically grown cells, albeit using somewhat smaller differencing intervals. The bands observed in the α region were at similar or identical wavelengths to those detected in spectra of cells grown under conditions of 'reduced aeration' (Poole *et al.*, 1980*a*), i.e. where the oxygen tension fell during growth to 10% of the saturated value. Numerical analysis of spectra of these aerobically grown cells did not reveal cytochrome b_{558} , which has been regarded as characteristic of an 'anaerobic-type' respiratory chain (Ashcroft & Haddock, 1975; Haddock & Jones, 1977). We have not identified the α bands detected, but it has been proposed (Shipp, 1972; Hendler *et al.*, 1975; Reid & Ingledew, 1979) that at least three different b -type cytochromes contribute to this region.

Shipp (1972) was unable to assign a specific band amongst those detected to cytochrome o , the CO-binding b -type cytochrome that is the major terminal oxidase in aerobically grown cells (Castor & Chance, 1959). Consequently, we subjected difference spectra (a sample that had been reduced and bubbled with CO *minus* an oxidized sample) to numerical analysis, but found no evidence for a decrease in the intensity of any band in the α region (results not shown). This is entirely consistent with the results of recent low-temperature flash photolysis experiments which showed that the ratio of the Soret absorbance to the α absorbance at 556 nm is high (approx. 22) and that the latter band is broad (Poole *et al.*, 1979; Scott *et al.*, 1981).

The α region of a reduced *minus* oxidized spectrum (not shown) of membrane particles isolated from aerobically grown cells was similar to that of intact cells (Fig. 2*a*) with a maximum at 555.5 nm. Numerical analysis using differencing intervals of 3.0, 2.6, 2.4 and 2.2 nm revealed only three components, at 547, 555.5 and 563.5 nm, whereas the use of smaller intervals showed the same five bands as those detected in whole cells (Fig. 2*b*). Preliminary evidence for the contribution of two sub-components to the 555.5 nm band came from experiments with membranes that had been washed with low ionic strength buffer

Fig. 2. Numerical analysis of the α regions of difference spectra of intact cells of *E. coli* grown under aerobic (a), oxygen-limited (c) and anaerobic (e) conditions. Spectra (a) and (c) are each the sum of five spectra, obtained by oxidizing the contents of the reference cuvette with H_2O_2 and reducing the contents of the sample cuvette with a few grains of $\text{Na}_2\text{S}_2\text{O}_4$ before freezing the cuvettes and recording the spectra at 77 K. Spectrum (e) is similar except that six spectra were summed. The respective fourth-order finite difference spectra (b), (d) and (f) were computed using differencing intervals of 1.8, 1.4, 1.2 and 1.0 nm. Spectra were scanned at 0.2 nm s^{-1} at a spectral bandwidth of 1.0 nm. The cell suspensions used for (a), (c) and (e) contained 50, 18.4 and $13.1 \text{ mg protein ml}^{-1}$, respectively.

(results not shown). The intensity of this band was diminished after washing and showed a split peak with maxima at 554 and 556 nm. This low ionic strength wash (Roisin & Kepes, 1973) has previously been shown to solubilize extensively a number of dehydrogenases and F_1ATPase (Poole & Haddock, 1974). *b*-type cytochromes that are loosely membrane-bound have been described by Hendler *et al.* (1975) and Reid & Ingledew (1979).

α absorption bands of oxygen-limited cells

Growth of *E. coli* under conditions of oxygen limitation results in the synthesis of large amounts of cytochromes a_1 and d (Poole *et al.*, 1981*a*), in addition to a cytochrome *o* (Rice & Hempfling, 1978; Poole *et al.*, 1981*a*) that is spectrally and kinetically distinct from the cytochrome *o* of aerobically grown cells (Poole & Chance, 1981). In fact, the concentrations of cytochromes a_1 and d in oxygen-limited cells were higher [0.047 and $0.334 \text{ nmol (mg protein)}^{-1}$, respectively] than in anaerobically grown cells [0.041 and $0.110 \text{ nmol (mg protein)}^{-1}$, respectively]. In aerobically grown cells, the concentration of cytochrome d was $0.018 \text{ nmol (mg protein)}^{-1}$; cytochrome a_1 was detectable but at a concentration too low to quantify. Thus, although oxygen-limited cells were not studied by Shipp (1972), it was of

interest to examine the *b*- and *c*-type cytochromes that are synthesized concomitantly with the alternative oxidases *d* and *a*₁.

Only two peaks, at about 556 and 558 nm, were detected in the α region of a reduced *minus* oxidized difference spectrum of cells grown under oxygen-limited conditions (Fig. 2*c*). Numerical analysis using the same differencing intervals as in Fig. 2(*b*) revealed only two major components, at 555.5 and 559 nm (Fig. 2*d*). Bands to either side of these prominent features were of much lower intensity than in the spectrum from aerobically grown cells (Fig. 2*b*). The use of larger differencing intervals (3.0, 2.6, 2.4 and 2.2 nm, results not shown) revealed further bands at 548 and 565 nm; presumably these bands were too broad to be detected at the smaller intervals used to obtain Fig. 2(*d*).

Numerical analysis of the α region of difference spectra (sample reduced and bubbled with CO *minus* an oxidized sample; not shown) revealed the same components as those shown in Fig. 2(*d*), again with no evidence for diminution of the intensity of either band that might suggest its identification as cytochrome *o*. The bands due to the reduced and CO-liganded forms were again presumably too small and/or broad to allow resolution using these differencing intervals. The CN⁻-binding properties of the cytochromes of cells grown under oxygen limitation were also investigated. In the α region of the CN⁻ + reduced *minus* reduced spectrum (not shown), troughs at 558 and 625 nm were observed. The 625 nm trough was due to cytochrome *d* whilst the 558 nm trough was presumably due to the CN⁻-binding *b*-type cytochrome observed by Pudek & Bragg (1974) that is distinct from cytochrome *o*.

α absorption bands of anaerobically grown cells

The α region of the reduced *minus* oxidized spectrum of cells grown anaerobically with fumarate as electron acceptor (Fig. 2*e*) showed two peaks, at 550.5 and 556 nm, with a shoulder at 558 nm. Numerical analysis of this spectrum revealed absorption bands at 548, 550.5, 555.5 and 559 nm (Fig. 2*f*). The first two bands were presumably due to two *c*-type cytochromes: the presence of two such cytochromes was inferred by Shipp (1972). The positions of peaks in the numerical analyses of spectra of anaerobically grown cells were clearly different from the positions in cells grown either aerobically or under oxygen-limited conditions. When the data for anaerobically grown cells were analysed at the same differencing intervals as used by Shipp (1972) (4.0, 3.0, 2.5 and 2.0 nm), components at 549, 557 and 559 nm were observed.

Numerical analysis of the spectrum of the difference between a CO-treated, reduced sample and an oxidized sample (not shown) showed the 550.5 nm component to be reduced in intensity by about 20%, suggesting the presence of a CO-binding *c*-type cytochrome. This is likely to be the component described as absorbing at 552 nm at 298 K by Barrett & Sinclair (1967). Other workers, however, have found no evidence for CO-binding *c*-type cytochromes in *E. coli* (Gray *et al.*, 1963; Fujita, 1966*a, b*; Fujita & Sato, 1963; Wimpenny *et al.*, 1963). Again, cytochrome *o* could not be detected using numerical analysis techniques. In contrast to reports of its presence (Trutko *et al.*, 1978), its constitutive synthesis (Haddock & Jones, 1977) and its detection by low-temperature flash photolysis (R. K. Poole, R. I. Scott & B. Chance, unpublished results) in anaerobically grown cells, Reid & Ingledew (1979) found no CO-binding *b*-type cytochromes under these conditions.

Changes in the Soret region revealed by numerical analysis

Numerical analysis of the Soret region of difference spectra of *E. coli* was not reported by Shipp (1972). The reduced *minus* oxidized difference spectrum of aerobically grown cells (Fig. 3*a*) showed a single peak at 426 nm. Numerical analysis (Fig. 3*b*) yielded major components at 430 and 438.5 nm. Although small, the bands at 417 and 424 nm were consistently observed in all spectra obtained of this region. The 438.5 nm component of aerobically grown cells was sharp, in contrast to the broad peak ranging from 438 to 443 nm in cells grown

The spectrum of cells grown anaerobically (Fig. 3e) showed a peak at 427 nm with shoulders at 418 and 440 nm. Analysis revealed a large peak at 417.5 nm (probably reflecting the increased concentration of *c*-type cytochrome; see Fig. 2e), a peak at 427.5 nm and a broad peak centred about 439.5 nm having a shoulder at about 442 nm. Although these peaks have not been rigorously identified, it is clear that the components present in cells grown aerobically and anaerobically are quite distinct, confirming the results obtained on inspection of the α region.

A semi-logarithmic plot of the amounts of cytochromes per cell detected by finite difference analysis of spectra of whole cells is shown as a function of mean cell volume in Fig. 4. The

Fig. 4. Accumulation of cytochromes during the cell cycle of aerobically grown cells. The cells from 4 l of an exponentially growing culture were fractionated into different size classes on a sucrose gradient in a zonal rotor. Finite difference analysis of spectra of cells at different stages in the cell cycle was carried out at differencing intervals of 3.0, 2.6, 2.4 and 2.2 nm, and the magnitudes of each component, expressed on a per cell basis, are plotted semi-logarithmically as a function of mean cell volume. The continuous lines fitted to the data for each component are those of best fit, the correlation coefficients, r , being 0.93 (563 nm), 0.91 (556 nm) and 0.80 (547 nm). The dashed lines represent the extent of two standard deviations from the fitted curves. The vertical bar on the right represents a doubling in amount of cytochrome per cell. The extent of the 'idealized' cell cycle (see text) is shown by the horizontal bar.

validity of using changes in mean cell volume as an indicator of cell age in fractions from a zonal rotor has been demonstrated previously (Scott *et al.*, 1980). The finite difference spectra of the different cell cycle fractions showed clearly resolved components at 547, 555.5 and 563.5 nm. The semi-logarithmic plots for each component are well fitted by straight lines; the small deviations from this pattern are ascribed to experimental error. The lines shown are those of best fit, but acceptable fits may be obtained through each of the three sets of data with parallel lines. The error inherent in the assay was estimated by dividing a cell suspension into seven portions and determining the cytochrome concentration per cell in each. The coefficients of variation for each component were 7% (563 nm), 6% (556 nm) and 6% (547 nm). Taken with the error inherent in the cell count determinations (3%), none of the experimental points can be considered to be significantly removed from the fitted lines.

The limits of an 'idealized' cell cycle calculated as described previously (Scott *et al.*, 1980) are also shown in Fig. 4. During this period, the concentration of cytochrome per cell for each component increased 1.60-fold (547 nm), 1.82-fold (556 nm) and 2.15-fold (563 nm). Other components detected in the β region at 525 and 536 nm also showed exponential increases during the cell cycle (not shown). Quantification of cytochromes using numerical analysis at smaller differencing intervals proved unsatisfactory as the coefficients of variation in peak height for the five components detected in the α region were excessively high.

DISCUSSION

A re-examination of the composite α band of cytochromes observed between about 540 and 570 nm in either intact cells of *E. coli* or in membranes derived therefrom has revealed the following: (1) in aerobically grown cells five components are present and are found at

Table 1. Comparison of the positions of absorption bands (nm) at 77 K resolved by fourth-order finite difference analysis, as determined in this paper and in a previous study (Shipp, 1972)

The data in columns 1, 2 and 3 are the wavelength maxima observed after fourth-order finite difference analysis of reduced *minus* oxidized difference spectra of cells grown under the conditions of aeration indicated. The α region was analysed at differencing intervals of 1.8, 1.4, 1.2 and 1.0 nm and the Soret region at intervals of 3.0, 2.6, 2.4 and 2.2 nm. The data in columns 4 and 5 were obtained by finite difference analysis at differencing intervals of 4.8, 3.6, 3.0 and 2.4 nm, and 4.0, 3.0, 2.5 and 2.0 nm, respectively; these are the same differencing intervals as used by Shipp (1972). The data in columns 6 and 7 are the absorption maxima obtained by Shipp (1972).

Data from this paper					Data from Shipp (1972)		
	1	2	3	4	5	6	7
	Aerobic	Oxygen-limited	Anaerobic	Aerobic (Shipp's differencing intervals)	Anaerobic (Shipp's differencing intervals)	Aerobic	Anaerobic
α region	548	—	548	546	549	548	549
	551.5	—	550.5	—	—	—	—
	—	—	—	—	—	552	553
	555.5*	555.5*	555.5*	555*	557*	556	556
	560	559†	559	—	559	560	561
	563	—	—	563	—	565	565
Soret region	417	417.5	417.5	—	—	—	—
	424	—	—	—	—	—	—
	—	427.5	427.5	—	—	—	—
	430	—	—	—	—	—	—
	438.5	439.5	439.5	—	—	—	—
	—	443.5	—	—	—	—	—

* The band at 555–556 nm probably includes a contribution from cytochrome *o* under all growth conditions and, in membranes from aerobically grown cells, also contains a loosely-bound component.

† CN⁻-binding component.

wavelengths close to those reported by Shipp (1972); (2) cytochrome *o* is not identified by its CO-binding properties in these numerical analyses; (3) the 555.5 nm component, which presumably includes cytochrome *o*, may consist of multiple components, which exhibit different degrees of resistance to removal from the membrane by washing with a buffer of low ionic strength [similar resolution can also be achieved by recording spectra of particles poised at different redox potentials (Reid & Ingledew, 1979; J. E. van Wielink, L. F. Oltman, F. J. Leeuwerik, J. A. de Hollander & A. H. Stouthamer, personal communication)]; (4) the components detectable by numerical analysis are dependent on the growth conditions employed. We have not been able to substantiate the claim made by Shipp (1972) that it is only the relative proportions of a fixed set of absorption bands that are altered. Examination of the Soret bands confirms the diversity of cytochromes synthesized under different growth conditions. The findings of the present paper are summarized and compared with those of Shipp (1972) in Table 1. Our results are more consistent with current schemes for the respiratory chain of *E. coli* which propose that spectrally, kinetically and potentiometrically distinct cytochromes are synthesized under different growth conditions (Haddock, 1980).

Although the results of Shipp (1972) are frequently cited as providing evidence for the complexity of cytochrome bands in *E. coli*, it has been difficult to relate the α bands reported by him to the components that have now been partly characterized with respect to their ligand-binding and potentiometric properties. In Table 2 we have attempted to correlate potentiometric and ligand-binding data on the cytochromes in aerobically grown cells;

Table 2. *Spectral, potentiometric and ligand-binding properties of the cytochromes in aerobically grown E. coli*

Wavelength maxima (nm) in difference spectra*		E_{m7} (mV)	Other properties	References
298 K	77 K			
552		+150	<i>c</i> -type; binds CO	Barrett & Sinclair (1967)
555			<i>b</i> - or <i>c</i> -type; binds CN^-	Pudek & Bragg (1974)
	556	+35		Pudek & Bragg (1976)
		-45		Kita <i>et al.</i> (1978)
		+260†	E_{m7} affected by CO (cyt. <i>o</i> ?)	Reid & Ingledew (1979)
		+80		
557.5			Detected	
			Detected	
			Purified as 'cyt. b_1 '	Deeb & Hager (1964)
	558	-50		Reid & Ingledew (1979)
		+165		Pudek & Bragg (1976)
			Detected	Downie & Cox (1978)
562			Not detected	Kita & Anraku (1981)
		+113		Itagaki & Hager (1966)
		+175-225	Soluble	Fujita (1966 <i>a</i>)
	562		Detected	Kita & Anraku (1981)
			Detected	Downie & Cox (1978)
	563	+260†		Reid & Ingledew (1979)

Hendler & Shrager (1979) and Hendler *et al.* (1975) also showed potentiometrically three components in the 'cytochrome b_1 ' peak but did not describe their absorption maxima.

* Dashed lines indicate that the two components are possibly the same. The continuous line indicates that the component absorbing at 562 nm at 298 K absorbs at 558 nm at 77 K (Itagaki & Hager, 1966).

† The component with E_{m7} of +260 mV appears to have a split α peak.

comparison with Table 1 shows that the major bands detectable by numerical analysis have been characterized independently. The 552 nm component detected at room temperature may correspond to either the 548 or 551.5 nm bands observed after numerical analysis. We have, however, obtained no evidence for ligand binding of these cytochromes (which are in low concentration in aerobically grown cells). The relationship between these components, the CO-binding *c*-type cytochromes of Barrett & Sinclair (1967) detected in aerobically grown cells and the CO-binding *c*-type cytochrome of anaerobically grown cells is not understood. An additional cytochrome c_{552} , first described by Gray *et al.* (1963), accepts reducing equivalents from formate dehydrogenase and may donate electrons to hydrogenase (O'Hara *et al.*, 1967) or, more likely, nitrite reductase (Douglas *et al.*, 1974). Since this cytochrome is periplasmic, however (for references, see Douglas *et al.*, 1974) it cannot account in full for the band seen in membrane particles at 548 or 551.5 nm. The band absorbing maximally at 555-556 nm is the most widely described component. There are radically different reports of the E_{m7} value associated with this component, and several workers have proposed that it contains more than one component. Our finding that part of the absorption band is removable to yield a heterogeneous peak provides further, but preliminary, evidence for the presence of multiple components. The 558 nm component in aerobically grown cells detected by some workers has not been resolved by us, possibly because it is at low concentration and/or its band width does not allow it to be resolved at the differencing intervals used. This band has been detected by using numerical analysis but only when applied in conjunction with potentiometric analysis (J. E. van Wielink, L. F. Oltman, F. J. Leeuwerik, J. A. de Hollander & A. H. Stouthamer, personal communication). The 563 nm component observed by Reid & Ingledew (1979), which may be a split α peak of the 556 nm band, could correspond to the peak observed in our analysis.

The high yield of cells obtained after zonal fractionation of exponentially growing cultures into different size classes allows numerical analysis to be applied to different cell cycle fractions. Ohki (1972) reported that the so-called 'cytochrome b_1 ' (absorbing maximally at 560 nm at 298 K) increased in a stepwise fashion during the cell cycle of *E. coli*. This finding can be criticized on several grounds for reasons given previously (Scott *et al.*, 1981). We have shown before that one component (cytochrome *o*, identified unequivocally by flash photolysis of the CO-liganded form) contributing to this band increases continuously through the cell cycle (Scott *et al.*, 1981); the present paper shows that all other detectable bands in this region accumulate in the same way. This lends support to the proposal that all membrane-associated components of the respiratory chain studied so far (including iron-sulphur clusters detectable by electron paramagnetic resonance; Poole *et al.*, 1981*b*) accumulate continuously throughout the cell cycle of *E. coli*.

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