Biochimica et Biophysica Acta, 292 (1973) 644–653

BBA 46512

KINETIC AND THERMODYNAMIC PROPERTIES OF MEMBRANE-BOUND CYTOCHROMES OF AEROBICALLY AND PHOTOSYNTHETICALLY GROWN *RHODOPSEUDOMONAS SPHEROIDES*

J. L. CONNELLY*, O. T. G. JONES, VENETIA A. SAUNDERS and D. W. YATES

Department of Biochemistry, University of Bristol, BS8 1TD (Great Britain)

(Received October 13th, 1972)

SUMMARY

A green mutant of *Rhodopseudomonas spheroides* was isolated in which spectroscopic measurements of the α-band region of cytochromes could be made. It was grown either aerobically or photosynthetically, and the membrane fractions prepared from cells of each type. Anaerobic potentiometric titration at 560 nm *minus* 542 nm showed the same three redox components, tentatively identified as *b*-type cytochromes, in membrane fractions from either type of cell. The mid-point potentials were approximately +185, +41 and −104 mV. In membranes from photosynthetically grown cells the major cytochrome form absorbing at 560 nm had a mid-point potential of +42 mV; in aerobically grown cells the major form had a potential of +185 mV. In both types of cell only one *c*-type cytochrome was found, with a mid-point potential of +295 mV. An *a*-type cytochrome was present only in aerobically-grown cells.

Substrate-reduced particles from these cells were mixed with air-saturated buffer in a stopped-flow spectrophotometer and the kinetics of oxidation of *b*- and *c*-type cytochromes were measured. The same two *b*-type components, reacting with pseudo first order kinetics, were detected in particles from both aerobically and photosynthetically grown cells ($t_{\frac{1}{2}}$ for oxidation 1.3 s and 0.13 s). The *c*-type cytochrome of particles from aerobically grown cells was oxidised with $t_{\frac{1}{2}}$ of 0.97 s; the *c*-type cytochrome of photosynthetic cells was oxidised faster, with $t_{\frac{1}{2}}$ of 0.27 s.

These observations have implications on the adaptive formation of electron transport systems that are discussed.

INTRODUCTION

The purple non-sulphur bacterium *Rhodopseudomonas spheroides* can grow aerobically if supplied with O_2 and organic substrates or, in the absence of O_2, can grow photosynthetically. For photosynthetic growth large structural changes take place in the organism, with the elaboration of many vesicles carrying the light-harvesting and photoreactive pigments. Some changes in the cytochrome content are

* Present address: Department of Biochemistry, University of North Dakota, Grand Forks, N.D. 58201, U.S.A.

also found. Particles from *R. spheroides* grown in either way are capable of respiration but only in aerobically grown forms is there evidence for an electron transport pathway with a terminal oxidase of the *a*-type; in the photosynthetically grown form the oxidase may be a cytochrome of the *o*-type[1,2,3]. Kikuchi and Motokawa[4] have suggested that the *a*-type oxidase is accompanied by a *c*-type cytochrome with a mid-point potential of +246 mV, such as is found in mitochrondria; there is no evidence for such a *c*-type cytochrome in photosynthetically grown cells[5].

In order to determine the changes in the nature and organisation of membrane-bound cytochromes following adaptation to heterotrophic growth we have determined the mid-point potentials of the cytochromes absorbing at 552 and 560 nm and have also measured the kinetics of oxidation of these cytochromes when anaerobic suspensions of particles are mixed with air-saturated buffer. These measurements show that alteration of the relative abundance of the *b*-type cytochromes, and in the reaction of the bulk cytochrome *c* with the terminal oxidase, can be induced by changes in growth conditions.

MATERIALS AND METHODS

Preparation of a green mutant of R. spheroides

This mutant, containing carotenoids absorbing light at shorter wavelength than those of the wild type, was prepared by treatment of cells of strain 2.4.1 with *N*-methyl-*N*-nitroso-*N*-nitroguanidine[6,7]. Photosynthetically-competent green cells were selected from isolated colonies on agar plates incubated anaerobically in the light. The rate of photosynthetic growth of this mutant was the same as for strain 2.4.1 (4–6 h generation time) and also the rates of aerobic growth were the same (3–3.5 h generation time). This mutant had the advantage of permitting spectroscopic observation of the cytochromes with little interference from carotenoids and was not liable to photodegradation in the presence of air.

Growth of cells

Cultures of the green mutant were either grown aerobically, in a vigorously aerated continuous culture apparatus[3], or anaerobically in the light in the medium of Sistrom[8]. Particles were prepared from cells disrupted in a French pressure cell, as described previously (ref. 3).

Spectrophotometry

The split-beam spectrophotometer used in this work has been described elsewhere[9] and the dual-wavelength spectrophotometer was essentially based upon the design of Chance[10].

Determination of mid-point potentials

The anaerobic procedures for the assay of oxidation–reduction mid-point potentials were those of Dutton *et al.*[11] using a stirred anaerobic cuvette, fitted with platinum and calomel electrodes, through which was bubbled a gentle stream of purified N_2. The wavelength pairs used in the dual wavelength spectrophotometer were: cytochrome *c*, 552 nm *minus* 542 nm; cytochrome *b*, 560 nm *minus* 542 nm. To obtain reliable measurements it was necessary to add redox mediators (see ref. 11)

to facilitate electron equilibration between the platinum electrode and the membrane-bound electron transport carriers.

Kinetic studies

The stopped-flow spectrophotometer used in these studies was essentially that described by Gutfreund[12,13]. This equipment had a dead time of 3 ms and an optical path length of 10 mm. The reciprocal dispersion of the monochromator was 2.6 nm per mm and the exit slits were fixed at 0.6 mm. Particles were allowed to become anaerobic in one syringe of the apparatus, due to respiratory activity following the addition of 2 mM sodium succinate. The time required for this anaerobic transition had been previously determined for each batch of particles by following the extent of reduction of cytochromes after substrate addition to particles contained in a cuvette of a split-beam spectrophotometer. The second syringe of the stopped-flow apparatus contained air-saturated buffer.

RESULTS

The spectroscopic properties of the cytochromes of the particulate fraction of the green mutant of *R. spheroides* when grown photosynthetically or aerobically are shown in Fig. 1. The very strong absorption of chlorophyll around 600 nm and of carotenoids at wavelengths below 520 nm limit spectroscopic studies of the photosynthetic form to the region between these wavelengths. In the anaerobic spectra, obtained after O_2 in the cuvette had been consumed, both the photosynthetic form and the aerobically grown form have relatively more reduced cytochrome *c* than reduced

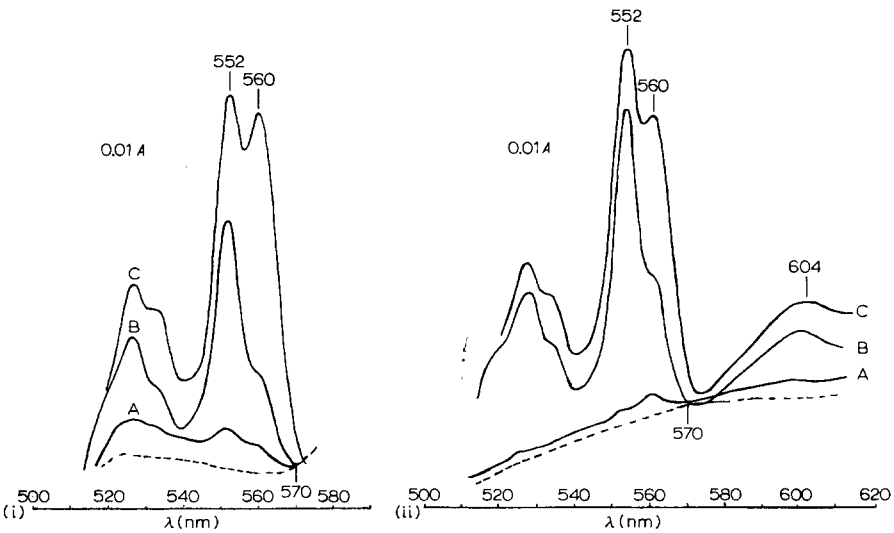


Fig. 1. Effect of addition of succinate and dithionite to particles prepared from (i) photosynthetically grown; (ii) aerobically grown *R. spheroides*. Particles ((i) 2 mg/ml protein or (ii) 3.5 mg/ml protein) were suspended in 10 mM Tris-HCl (pH 7.5); 2 mM succinate was added to test cuvette and spectra recorded at room temperature. Trace A was recorded immediately after adding succinate; Trace B (i) 15 min later; (ii) 6 min later. Trace C was recorded after addition of dithionite to test cuvette. – – – –, baseline.

b. The addition of dithionite causes further reduction, mostly of *b*-type cytochrome, and gives a ratio of *b*/*c* approximately equal to 1:1. In particles from the aerobically grown *R. spheroides* the absorption maximum at 606 nm due to reduced cytochrome oxidase can be clearly seen in the anaerobic steady state.

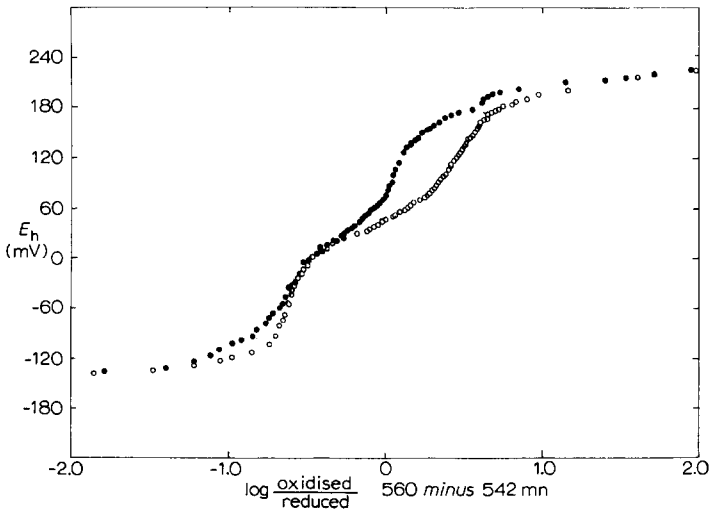


Fig. 2. Potentiometric titration of the cytochromes of particles from aerobically grown and photosynthetically grown *R. spheroides* measured at 560 nm *minus* 542 nm. Particles (approx. 2 mg/ml protein) were suspended in 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, pH 7.0. 10 μM phenazine methosulphate, 10 μM phenazine ethosulphate, 7 μM pyocyanine, 10 μM diaminodurol and 10 μM 2-hydroxy-1,4-naphthoquinone were present as mediators. Oxidation–reduction potentials were made more negative by successive additions of succinate, NADH and dithionite. Potentials were made more positive by adding $K_3Fe(CN)_6$. ●, titration of particles from aerobically grown cells; ○, titration of particles from photosynthetically grown cells.

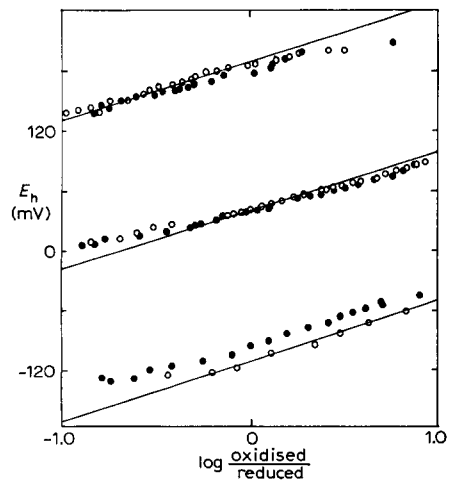


Fig. 3. Replot of the data from Fig. 2, showing the resolution of the curves into three components. The line drawn through the points is a theoretical $n = 1$ line derived from the Nernst equation.

A dark titration of the cytochromes of photosynthetically grown *R. spheroides* carried out by Dutton and Jackson[5] revealed three cytochromes considered to be of the *b*-type, with absorption maxima at 558–559, 560 and 564 nm; two *b*-type cytochromes were also resolved by Jones[14] using difference spectroscopy at 77 °K. A dark titration at 560 nm confirmed the presence of the three components described previously in photosynthetically grown cells[5] and showed that much less of the cytochrome with a mid-point potential around +42 mV was present in particles from aerobically grown cells (Fig. 2). Of the total cytochrome reduced when the potential fell from +240 mV to −160 mV in photosynthetic cells about 60% was the cytochrome with a mid-point potential near +40 mV and only about 34% in aerobic cells. The data from the titration curve of Fig. 2 were replotted after the curve had been resolved into three components and the experimental points fiitted well to the theoretical $n=1$ lines drawn (Fig. 3). The calculated midpoint potentials (pH 7.0) for the cytochromes, and ratio of concentrations are given in Table I.

TABLE I

MID-POINT POTENTIALS AND RATIOS OF CYTOCHROMES OF PARTICLES PREPARED FROM AEROBICALLY AND PHOTOSYNTHETICALLY GROWN *R. SPHEROIDES*

Measurements were made at pH 7.0.

Cytochromes	*Growth conditions*	*Measured* E_0 *(mV)*	*Proportion of total cytochrome change (%)*
b-type (560 nm)	Aerobic	+185	48
		+ 41	34
		−104	18
	Photosynthethic	+189	29
		+ 42	57
		−112	14
c-type (552 nm)	Aerobic	+295	92
	Photosynthetic	+295	93

Titration at 552 nm from +350 mV to −50 mV gave for each type of particle only one component, with a mid-point potential of +295 mV. Small absorbance changes at low potential were found but were not further characterised. They may arise from minor cytochrome *c* components such as cytochrome c_3, described by Meyer *et al.*[15] or from interference by cytochrome *b* changes.

Analysis of the kinetics at 560 nm when substrate-anaerobic particles were mixed with aerated buffer in the stopped-flow spectrophotometer suggested the presence of at least two components that were rapidly oxidised (Fig. 4a). These were resolved into two components on replotting, assuming that the oxidations were pseudo first order reactions (Fig. 4b). Apparently the same two components were present in cells grown aerobically or photosynthetically, with $t_{\frac{1}{2}}$ for oxidation of approx. 130 ms and 1.3 s.

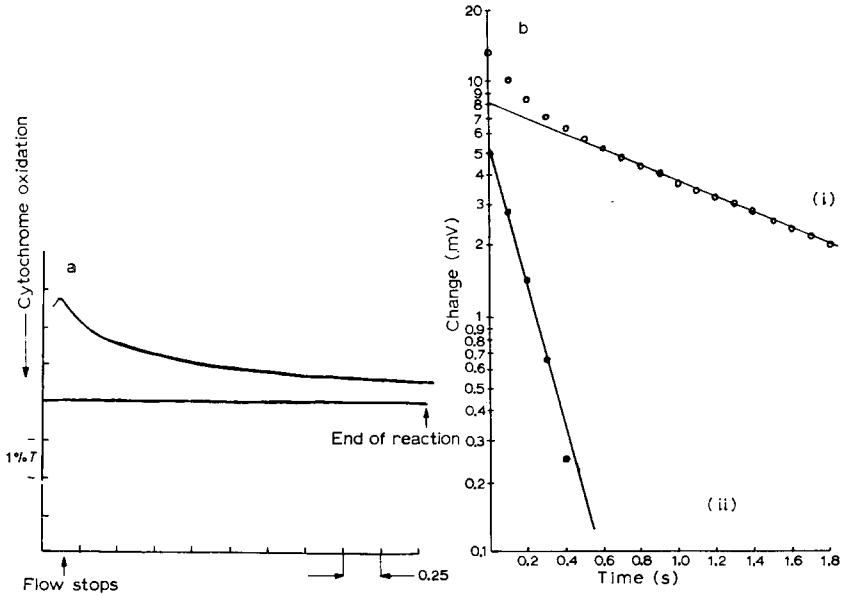


Fig. 4. (a) Kinetics of oxidation at 560 nm in particles prepared from photosynthetically grown *R. spheroides*. Particles (6 mg/ml protein) in 10 mM Tris–HCl (pH 7.5) were allowed to become anaerobic with 2 mM succinate and then mixed with an equal volume of air-saturated 10 mM Tris–HCl (pH 7.5) in the stopped-flow spectrophotometer. The time constant was 8 ms. (b) Replot of the data obtained from the trace shown in (a). $t_{\frac{1}{2}}$= (i) 1.0 s; (ii) 0.1 s; k= (i) 0.69 s^{-1}; (ii) 6.9 s^{-1}.

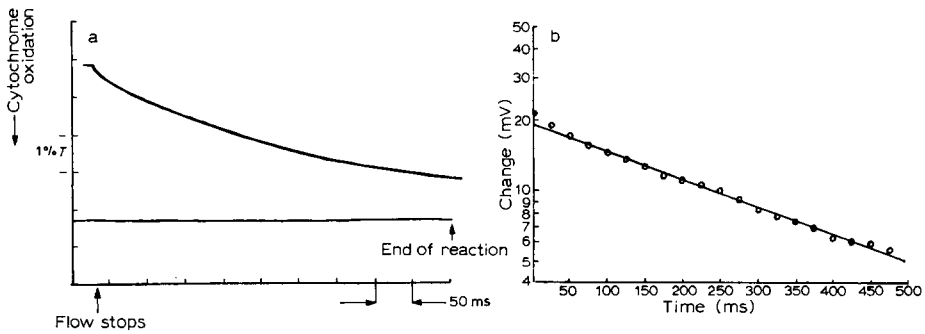


Fig. 5. (a) Kinetics of oxidation at 552 nm of particles prepared from photosynthetically grown *R. spheroides*. Experimental conditions were as described in Fig. 4a. (b) Replot of the reaction shown in (a). $t_{\frac{1}{2}}$= 0.26 s. k= 2.65 s^{-1}.

The kinetics of changes induced at 552 nm by oxygenation were simpler (Fig. 5a) and the replots, assuming pseudo first order reactions, gave only one component (Fig. 5b) in both aerobic and photosynthetic preparations, but the $t_{\frac{1}{2}}$ for the oxidation was significantly slower in the aerobic preparation. For the changes at 552 nm, replots by the method of Guggenheim[16] verified the constants obtained from standard replots. A summary of the constants obtained from a series of determinations is given in Table II. There was, in some runs, a suggestion that a very fast reacting component was present but the kinetics of this component were such that

TABLE II

SUMMARY OF KINETICS OF OXIDATION OF CYTOCHROMES IN PARTICLES FROM PHOTOSYNTHETICALLY GROWN AND AEROBICALLY GROWN *R. SPHEROIDES*

Particles	*λ (nm) of observation*	$t_{\frac{1}{2}}$ *(s)*	k (s^{-1})
Photosynthetic	552	0.27 ±0.04	2.5
	560	1.3 ±0.37	0.53
		0.123 ±0.02	5.6
Aerobic	552	0.97 ±0.1	0.71
	560	1.32 ±0.2	0.52
		0.13 ±0.04	5.31

it was difficult to resolve from a mixing artefact. In calculating the extent of transmission change, confirmation of the measured end of reaction was obtained from a second scan at slow speed.

A number of control experiments confirmed that the stopped flow measurements were not artefactual. No changes were observed at 570 nm, the isosbestic point for the transition between the aerobic steady state and anaerobic steady state (see Fig. 1), and no changes were detected when particles reduced with dithionite were mixed with buffer containing dithionite. When antimycin A was added to particles in the anaerobic steady state, only the kinetics of oxidation of the fast 560 nm component were unaffected; the slow reaction was almost completely abolished in both types of particle (Fig. 6). No such effect of antimycin A was found at 552 nm. A series of oxygen electrode determinations showed that antimycin A, at the concentrations used, gave inhibitions of succinate oxidation of about 65% for particles from both aerobic and photosynthetic cells.

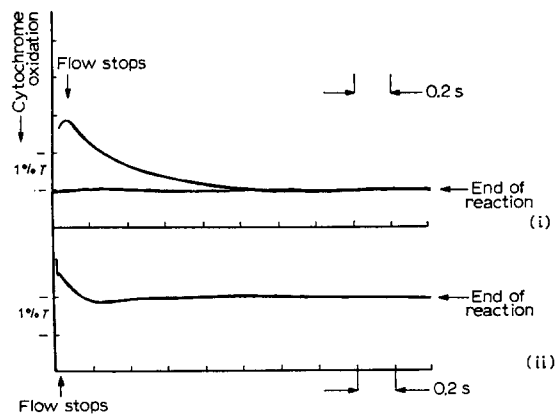


Fig. 6. Effect of antimycin A on the kinetics of oxidation at 560 nm in particles prepared from (i) photosynthetically grown *R. spheroides*. Experimental conditions were as described in Figs 4a and 5a, respectively, except that 4 μM antimycin A was added after anaerobiosis had been attained.

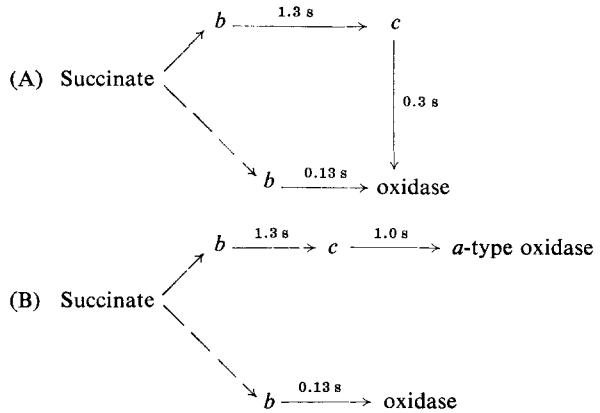
DISCUSSION

The titrations of the cytochromes absorbing at 560 nm in particles from photosynthetically-grown cells gave three components with mid-point potentials that are in reasonable agreement with those previously published by Dutton and Jackson[5,17], although the relative amounts of each component differ from their values. This is because our titrations were made at 560–542 nm, and theirs at 566–540 nm. The absorption maximum of the low potential component is near 566 nm, that of middle potential carrier is at 560 nm and consequently the wavelength selected for titrations will very much influence the measured ratios. From measurement of the *b*-cytochrome reactions, following a laser flash, Dutton and Jackson concluded that a *b* cytochrome with a mid-potential of +50 mV (b_{50}) ultimately accepts electrons from photoreduced X, the primary electron acceptor in photosynthesis, and so has a central role in energy conservation in a cyclic light driven electron flow, and that b_{155} is involved in a non-cyclic mechanism for feeding electrons into the cyclic system, *via* cytochrome c_{295}. The role of the long wavelength, low potential b_{-90} was unclear. Our results provide support for the scheme outlined above. During aerobic growth the proportion of the b_{42} decreased, suggesting that this component is not important in aerobic growth but is necessary for photosynthesis. (Our conditions for aerobic growth did not completely abolish the synthesis of photosynthetically active pigments; the chlorophyll content was reduced to about 5% and actinic illumination induced a photo-oxidation of cytochrome *c*, and some reduction of a cytochrome *b*, see also ref. 3.)

Although it is possible to detect a minor cytochrome *c* component in membrane fractions by spectroscopy at 77 °K (ref. 14) this was not resolved by redox titration. It appears that cytochrome c_{295} plays an important role in both cyclic electron flow and in dark, respiration-linked electron flow in these membrane preparations. Other soluble cytochromes of the *c*-type, that are present in whole cells of aerobically grown *R. spheroides*[3,18], are lost in the supernatant fraction. Similarly, kinetic studies of cytochrome *c* oxidation in the particles revealed only one component in cells grown either aerobically or photosynthetically, although, again, the presence of minor components cannot be excluded. It appears that when adapting from photosynthetic to aerobic conditions the same cytochromes *b* and *c* are used in synthesising new membrane-bound electron transport systems.

In kinetic studies with whole cells of photosynthetically grown *R. spheroides*, Chance and his co-workers[19] found that a pigment absorbing maximally at 428 nm had many properties expected of an oxidase. The pseudo first order rate constant was about 0.5 s^{-1}, with no evidence for biphasic kinetics. In particles from cells of both types we find that, at 560 nm, there is a component that reacts with a value for *k* of about 0.5 s^{-1} but, in addition, a significantly faster component reacts, with $k = 5.3\ s^{-1}$. This fast component, which represents about 30–40% of the oxidisable material at 560 nm, is unlikely to be an oxidase of the *o*-type since much more CO-reactive pigment would then be expected in difference spectra and also the midpoint potentials of the major components at 560 nm are well below that of the cytochrome *c*. We tentatively suggest that a minor component is present that acts as an oxidase. The rates of oxidation are such that this oxidase could not be cytochrome *cc'* (see ref. 19). The kinetics of *b*-cytochrome oxidation in particles from aerobically-grown cells and

photo synthetically-grown cells did not differ significantly; the major difference between the particles was that the oxidation of cytochrome *c* was slower in aerobic particles. This suggests that the orientation of cytochrome *c* to its original oxidase has changed, probably as a result of the development of the new *a*-type oxidase and modification of the photosynthetic electron flow system, and it is no longer in a position to react directly with the fast oxidase. A number of schemes could be invoked to explain these kinetics but we limit these to the bare outline given below in Scheme I.



Scheme 1. (A) Oxidase system of photosynthetically-grown organisms. (B) Oxidase system of aerobically-grown organisms. The times given on the arrows refer to the $t_{\frac{1}{2}}$ of oxidation.

The *b* and *c* components with similar rate constants found in the aerobic form need not give complex kinetics, since the *c* is present in a large excess compared with the *b*. The fast *b* component may be responsible for the antimycin-resistant component of respiration and its oxidase could be some autoxidisable component normally present between the fast *b* and cytochrome *c*, if these were both components of the cyclic electron transport system, *i.e.* if the fast *b* is b_{42}. It is unlikely that the low potential *b* would be detected in the stopped-flow experiments since conditions in the syringes would be insufficiently reducing to achieve the E_h needed for its reduction.

In summary, our results indicate that when *R. spheroides* undergoes the major structural and functional changes associated with adaptation to the heterotrophic existence it incorporates at least one existing *b* cytochrome and one existing *c* cytochrome into a respiratory electron pathway and that these two cytochromes retain a similar orientation to each other, although a new terminal oxidase has been developed. A *b* cytochrome with a mid-point potential of +42 mV is synthesised in smaller amounts when the cells are grown aerobically.

ACKNOWLEDGEMENTS

We are grateful to the Science Research Council for a Research Grant and a Research Scholarship (to V. A. Saunders). J. L. Connelly gratefully acknowledges support provided by U.S. Public Health Service Research Career Programme Award 1-K3-GM-7028-05. We thank Professor H. Gutfreund for interesting discussions on this work and the use of his stopped-flow spectrophotometer.

REFERENCES

1 Kikuchi, G., Saito, Y. and Motokawa, Y. (1965) *Biochim. Biophys. Acta* 95, 1–14
2 Motokawa, Y. and Kikuchi, G. (1966) *Biochim. Biophys. Acta* 120, 274–281
3 Jones, O. T. G. and Whale, F. R. (1970) *Biochim. Biophys. Acta* 223, 146–157
4 Kikuchi, G. and Motokawa, Y. (1970) in *Structure and Function of Cytochromes* (Okunuki, K., Kamen, M. D. and Sekuzu, I., eds), pp. 174–181, University of Tokyo Press, Tokyo
5 Dutton, P. L. and Jackson, J. B. (1972) *Eur. J. Biochem.* 30, 445–510
6 Adelberg, E. A., Mendel, M. and Chein Ching Chein, G. (1965) *Biochem. Biophys. Res. Commun.* 18, 788–795
7 Lascelles, J. (1966) *Biochem. J.* 100, 175–183
8 Sistrom, W. R. (1960) *J. Gen. Microbiol.* 22, 778–785
9 Jones, O. T. G. and Saunders, V. A. (1972) *Biochim. Biophys. Acta* 275, 427–436
10 Chance, B. (1951) *Rev. Sci. Instrum.* 22, 634–638
11 Dutton, P. L., Wilson, D. F. and Lee, C. P. (1970) *Biochemistry* 9, 5077–5082
12 Gutfreund, H. (1965) *An Introduction to the Study of Enzymes* pp. 123–141, Blackwell Scientific Publications, Oxford and Edinburgh
13 Stinson, R. A. and Gutfreund, H. (1971) *Biochem. J.* 121, 235–240
14 Jones, O. T. G. (1969) *Biochem. J.* 141, 793–799
15 Meyer, T. E., Bartsch, R. G. and Kamen, M. D. (1971) *Biochim. Biophys. Acta* 245, 453–464
16 Guggenheim, E. A. (1926) *Philos. Mag.* 2, 538–544
17 Dutton, P. L. and Jackson, J. B. (1972) *Proc. 2nd Int. Congr. on Photosynthesis, Stresa, 1971*, pp. 995–1008, Junk N.V. Publishers, The Hague
18 Orlando, J. A. (1962) *Biochim. Biophys. Acta* 57, 373–375
19 Chance, B., Horio, T., Kamen, M. D. and Taniguchi, S. (1966) *Biochim. Biophys. Acta* 112, 1–7