Biochimica et Biophysica Acta, 440 (1976) 412–428

BBA 47127

RESPIRATORY COMPONENTS AND OXIDASE ACTIVITIES IN *ALCALIGENES EUTROPHUS*

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(Received January 9th, 1976)

SUMMARY

1. Cells of the hydrogen bacterium *Alcaligenes eutrophus* are broken by gentle lysis using lysozyme treatment in hypertonic sucrose followed by osmotic shock. By this method, 93 % of the in vivo activity of the H_2 oxidase is recovered and the ATPase remains particle bound. In contrast, cell disruption in a French pressure cell diminishes the in vivo activity of the H_2 oxidase by 50 % and solubilizes the bulk of the ATPase.

2. The bacterium contains a periplasmic cytochrome *c* with bands at 418, 521 and 550 nm (difference spectrum). In addition to cytochrome aa_3, *b*-560, *c*-553 and *o*, low temperature difference spectra of membranes show the presence of two further cytochromes (shoulders at 551 and 553 nm).

3. The unsupplemented membrane fraction catalyses the oxidation of hydrogen, NADH, NADPH, succinate, formate and endogenous substrate (NAD linked) at rates 2–3-fold higher than membranes obtained from cells disrupted in a French pressure cell. With the exception of the H_2 oxidase all oxidase activities in lysozyme membranes are sensitive to carbonylcyanide *m*-chlorophenylhydrazone (20–100 % stimulation of oxygen uptake).

4. The cytoplasmic fraction contains a *B*-type cytochrome with absorption maxima at 436 and 560 nm, capable of combining with CO; it contains non-covalently bound protohaem. In alkaline solutions a spectral transition to the haemochrome type with bands at 423, 526 and 556 nm occurs. The addition of NADH to an aerobic suspension of this cytochrome elicits new absorption maxima at 418, 545 and 577 nm (difference spectrum), which are believed to represent an oxygenated form of the reduced cytochrome.

INTRODUCTION

Hydrogen bacteria are characterized by their ability to grow chemoautotrophically using hydrogen as electron donor for their respiratory chain and CO_2 as carbon

Abbreviations: HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; CCCP: carbonylcyanide *m*-chlorophenylhydrazone.

source. The physiological group comprises a multitude of bacteria belonging to different taxomonic groups. The majority of investigations on the respiratory chain and energy-generating system of these bacteria have been done with gram-negative bacteria which belong to the genera *Pseudomonas* [1] and *Alcaligenes* [2–4]. The enzyme system involved in the oxyhydrogen reaction is bound to the cytoplasmic membrane. Flavoproteins, cytochromes *b*, *c*, aa_3 and *o* have been found [1–4], and ATP production by oxidative phosphorylation has been demonstrated with intact cells [5, 6] or crude extracts [1, 3, 7].

Unfortunately, studies concerning the respiratory chain in hydrogen bacteria have been carried out using rough mechanical methods of cell breakage. Inherent in such methods are a number of disadvantages, including (1) the difficulty in discerning the distribution of respiratory components within the cell structure (e.g. periplasmic space, cytoplasma ,membranes), (2) the possible damage to the membrane structure, which makes valid interpretation of the results difficult. Therefore, the work to be described was aimed to obtain cytoplasmic membranes, the biological activity of which was well preserved. Intact cells and osmotically disrupted spheroplasts as well as periplasmic space, membranes and cytoplasmic extracts were used to study the spatial distribution of various components of the respiratory chain and of oxidase activities within the cell. Evidence is presented for a soluble haemoprotein in *Alcaligenes eutrophus* which has not been described before.

METHODS

Growth of organism

A. eutrophus H 16 (= *Hydrogenomonas eutropha* H 16, ATCC 17699, German Collection of Microorganisms (DSM) 428) was grown in a mineral medium [8] supplemented with 0.05 % $NaHCO_3$ at 30 °C in a 10-l fermenter (Biostat, Braun, Germany) at 600 rev./min. At the beginning of growth, the gas atmosphere consisted of 80 % H_2, 10 % CO_2 and 10 % O_2; the gas flux was 500 ml/min. The cells grew exponentially without a lag phase ($\mu = 0.28\ h^{-1}$). When an absorbance (436 nm) of 2–4 was reached, the oxygen concentration in the atmosphere was increased to 15 % on account of the hydrogen concentration. During the exponential growth phase, the respiratory activity of the cells (manometric determination of the hydrogen oxidase activity) remained constant; it increased at the beginning of the stationary phase. The culture was harvested at mid-log phase ($A = 7$), centrifuged and washed in 50 mM Tris · HCl buffer (pH 7.4). Cells grown and prepared as described had reproducible respiratory activities and cytochrome contents.

Preparation of cell fractions

(a) *Lysozyme method.* The procedure for cell disruption, as outlined in Fig. 1, was effective only with freshly harvested cells. Cells were suspended in 10 mM Tris · HCl (pH 7.8) containing 0.5 M sucrose and 5 mM EDTA (1 g cells (wet weight)/10 ml buffer). The suspension was stirred for 10 min at 30 °C before addition of lysozyme (20 mg/g wet weight). The formation of spheroplasts was followed by diluting samples of the cell suspension in 100 vols. distilled water and by measuring the absorbance of this osmotically shocked lysate. After 30 min of incubation, 99 % of the cells were transformed to spheroplasts, as determined microscopically. The spheroplast suspen-

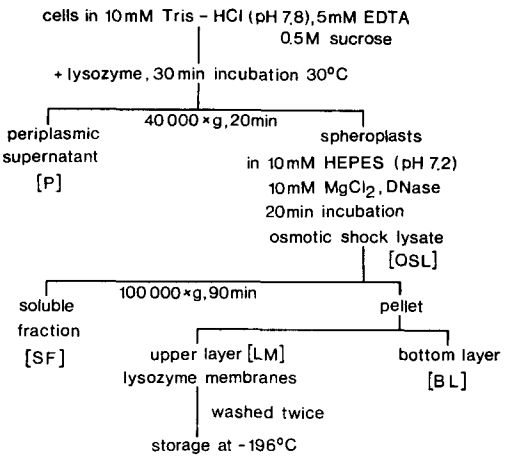

Fig. 1. Cell fractionation of *A. eutrophus* by the lysozyme method. Details are given in the text.

sion was centrifuged at 40 000 × *g* for 20 min. The supernatant fraction obtained will hereafter be referred to as periplasmic supernatant. The spheroplast pellet was homogenized in 10 mM HEPES/KOH buffer (pH 7.2), at 10 ml/g cells (wet wt.), and 10 mM $MgCl_2$ and DNAase (10 μg/g cells wet wt.) were added. After stirring at 20 °C for 20 min, the osmotically shocked lysate (referred to as OSL) was fractionated into particulate and supernatant fractions by centrifugation at 100 000 × *g* for 40 min. The particulate pellet consisted of two layers, an upper reddish one (lysozyme membranes) and a hard whitish bottom layer which was discarded. The lysozyme membrane fraction was carefully removed with a glas rod, washed twice in 50 mM HEPES/KOH (pH 7.2) and stored in small portions (1 ml, 15 mg protein/ml) under liquid nitrogen. The supernatant (SF) was recentrifuged to remove small particles.

(b) *Cell disruption by the french pressure cell.* Harvested cells were suspended in 50 mM HEPES/KOH (pH 7.2), DNAase (50 μg/g cells wet wt.) was added, and the mixture was passed once through a French pressure cell (Aminco, U.S.A.) at 1500 kP/cm^2. The disrupted cell suspension was fractionated into particulate and supernatant fractions by centrifugation at 120 000 × *g* for 90 min. The upper red membrane layer was removed and treated in the manner described above.

Electron microscopy

Thin sections were made from glutaraldehyde- and OsO_4-prefixed samples embedded in a mixture of plastic material [9]. The sections floating on thymol-containing water were picked up with carbon-coated Formvar grids and stained with uranyl acetate (2 % in water, pH 5) for 10 min and lead citrate (10 % in water, pH 12) for 5 min [10]. Electron microscopy was carried out with a Philips electron microscope EM 301 at primary magnifications of 7160 × or 71 000 ×.

Enzyme assays

Oxidase activities were measured at 25 °C using a Clark type oxygen electrode (Rank, Bottisham, England). In order to exclude the lag phase of the H_2 oxidase activity, the membrane suspension was bubbled with H_2 for 10 min at 25 °C before

injection into the reaction vessel. The electrode was calibrated with air-saturated distilled water assumed to contain 253 nmol oxygen/ml at 25 °C. The membrane-bound hydrogenase (hydrogen: methylene blue oxidoreductase) activity was measured manometrically at 30 °C in 50 mM potassium phosphate buffer (pH 7.0) with methylene blue as electron acceptor. The vessels were gassed for 15 min with CO_2- and O_2-free H_2 and the reaction started by tipping the methylene blue from the side arm into the main compartment (final concentration: 4.5 mM).

The soluble hydrogenase (EC 1.12.1.2) activity was determined photometrically at 25 °C in 1-cm cuvettes containing 50 mM Tris · HCl (pH 7.0, H_2-saturated), 1 mM NAD and the enzyme sample in a final volume of 3 ml. The reduction of NAD was followed.

The malate dehydrogenase (EC 1.1.1.37) activity was measured photometrically at 25 °C. The reaction mixture contained 50 mM Tris · HCl (pH 7.4), 5 mM oxaloacetate, 1 mM NADH and the enzyme sample in a final volume of 3 ml. The oxidation of NADH was followed.

The assay for the ATPase (EC 3.6.1.4) activity contained 50 mM Tris · HCl (pH 7.5), 5 mM NaATP, 4 mM sodium phosphoenolpyruvate, 5 mM $MgCl_2$, 30 μg pyruvate kinase and 0.4 mg membrane protein in a final volume of 1 ml. The reaction was started by the addition of ATP and stopped after a 10-min incubation at 30 °C by adding 1 ml of 10 % trichloroacetic acid. The precipitated protein was removed by centrifugation. 1 ml of the supernatant was used for the determination of inorganic phosphate by the method of Taussky and Shorr [11]. The control was incubated without substrate; ATP was added immediately after the addition of trichloroacetic acid.

Peroxidase (EC 1.11.1.7) activity was assayed according to Püttner [12].

Spectrophotometric techniques and cytochrome identification

Cytochrome spectra at room temperature were recorded on a Zeiss DMR 21 split-beam spectrophotometer using cuvettes of 1-cm light path. Dilutions of the samples were made in 50 mM potassium phosphate (pH 7.0) and the reduced minus oxidized difference spectra were obtained by reducing one cuvette with a few grains of dithionite. The reference cuvette was oxidized by addition of ferricyanide (periplasmic fraction) or by vigorous aeration (cytoplasmic fraction, membranes). CO difference spectra were obtained by reducing the samples as described above and bubbling CO for 60 s through one cuvette. The spectra were recorded after 5 min of incubation with CO in the dark. To record difference spectra at −196 °C, a split-beam spectrophotometer with low temperature equipment of the Max Planck Institut für Ernährungsphysiologie, Dortmund, was used. Samples were measured in cuvettes of 2-mm light path using the single freeze technique of Bonner [13] in the presence of 50 % glycerol.

Cytochromes were identified from α-peaks in their difference spectra [14]. The concentrations were estimated from dithionite-reduced minus oxidized difference spectra using $\varepsilon_{mM} = 24$ for cytochrome aa_3 [15], $\varepsilon_{mM} = 20$ for *b*-type cytochrome [16] and $\varepsilon_{mM} = 19$ for *c*-type cytochrome [16]. Cytochrome *o* was estimated from the dithionite-reduced plus CO minus dithionite-reduced difference spectra using $\varepsilon_{mM} = 170$ (peak–trough) according to Daniel [17].

The acid acetone extraction of protohaem and the formation of pyridine

haemochromes were carried out as described by Weston and Knowles [18]. The haems present in the extract and residue were identified by their difference spectra [14].

Quinone determination

The extraction procedure, identification and quantitative determination of quinones were performed using the method and extinction coefficients described by Kröger et al. [19]. Separation of the extracted ubiquinone and menaquinone (120 mg membrane protein extracted) was achieved by thin-layer chromatography on silica gel plates (Merck, Germany; 0.25 mm). The solvent system consisted of light petroleum/ethylether (16 : 4, by vol.), the chromatogram was developed for 30 min [4]. The quinones, identified by comparison to the references Q_{10} and vitamin K-1 were scraped off the plate, dissolved in acetone and separated from silica gel by repeated centrifugation.

DEAE column chromatography

20 ml (400 mg protein) of the soluble fraction, obtained from a cell disruption by means of the French pressure cell, were applied to a DEAE-Sephadex A-50 column (2.5 × 35 cm), which had been equilibrated with 20 mM potassium phosphate (pH 7.0). The column was washed with 100 ml of the above buffer and the protein eluted with a linear gradient of 0.0–0.5 M KCl in 600 ml equilibrium buffer. 200 fractions of approx. 3 ml each were collected at an average flow rate of 25 ml/h. The *B*-type cytochrome was identified by its difference spectrum. The fractions 125–145 were pooled, concentrated by ultrafiltration to a volume of 15 ml and dialyzed over-night against 20 mM potassium phosphate (pH 7.0).

Protein was determined by the biuret method [20].

Chemicals

All enzymes and biochemical substrates were purchased from Boehringer, Mannheim, Germany. HEPES buffer and lysozyme were obtained from Serva, Heidelberg, Germany, and CCCP from Calbiochem. Inc., Los Angeles, U.S.A. All other chemicals were purchased from Merck, Darmstadt, Germany.

RESULTS

Gentle cell lysis was achieved by lysozyme treatment in hypertonic sucrose followed by osmotic shock of the spheroplasts, as illustrated in Fig. 1 [21].

Although *A. eutrophus* is a Gram-negative bacterium, lysozyme treatment was effective; spheroplasting occurred even in the absence of EDTA, though with some delay. Since the presence of EDTA during the spheroplast formation had no effect on the respiratory activities of the membranes, the lysozyme treatment was carried out in sucrose media containing 5 mM EDTA.

Distribution of cell components among the cell fractions

The distribution of particulate and soluble components among the cell fractions is shown in Table I. The H_2 oxidase activity was a very sensitive indicator for membrane damage; in the osmotically shocked lysate the enzyme retained 93 % of its

TABLE I

DISTRIBUTION OF ENZYMES, PROTEIN AND *c*-TYPE CYTOCHROME IN CELL FRACTIONS OF *A. EUTROPHUS*

Fractions were prepared from intact cells by the lysis procedure illustrated in Fig. 1. The determination of enzyme activities and cytochrome content were as described under Methods. The values given represent the average of two different preparations.

Fraction	Percent of total activity				Percent of total content	
	H_2 oxidase	ATPase	Hydrogenase (soluble)	Malate dehydrogenase	Cytochrome *c* [b]	Protein
Cells	100		100 [a]	100 [a]	100	100
P	< 1	2	< 1	< 1	19	n.d. [c]
OSL	93	100	98	96	82	86
SF	< 1	7	93	91	5	33
LM+BL		90	< 1 [a]	< 1 [a]	73	
LM	76					23
BL	18					34

[a] Fractions were sonicated (15 s/ml) to allow the determination of enzyme activity.
[b] $Na_2S_2O_4$ reduced.
[c] Accurate value could not be obtained due to the presence of large amounts of lysozyme.

in vivo activity; 76 % of the original activity was recovered in the lysozyme membrane fraction, and the remainder of the activity (18 %) was discarded with the bottom layer. For comparison, cell disruption in the French pressure cell diminished the in vivo activity by 50 %, and ultrasonic treatment of the cells resulted in an activity loss of 70 %.

The procedure used for cell breakage strongly effected the distribution of ATPase activity. Whereas the bulk of the enzyme was solubilized by the use of the French pressure cell for cell disruption (specific activity of the membranes: 4 nmol P_i/min per mg protein), the enzyme remained in the particulate fractions obtained by the lysozyme method (Table I). 90 % of the measurable ATPase activity in the osmotically shocked lysate was recovered in the membraneous fractions (the ATPase is accessible only in inside-out vesicles); the specific activity in the lysozyme membrane fraction varied between 70 and 100 nmol P_i/min per mg protein in different preparations.

Spectrophotometric studies of the periplasmic fraction, the separation of which is only possible by non-mechanical cell disruption, revealed the presence of a cytochrome with peaks at 418, 521 and 550 nm in the difference spectrum (Fig. 2, curve A). Acid acetone extraction of the lyophilized periplasmic fraction, and formation of the pyridine haemochromes showed the haem to be of the *C*-type (414, 520, 549 nm). Low temperature difference spectra showed a single α-peak at 547 nm. The cytochrome was able to react with CO (413, 536 and 562 nm, troughs at 423 and 551 nm, Fig. 2, curve C), but the reaction was sluggish, and because of the low absorbance of the CO complex, the ability to bind CO is not believed to be of physiological significance. The periplasmic nature of this cytochrome was substantiated by comparison to the distribution of cytoplasmic enzymes among the cell fractions (Table I). No soluble NAD-specific hydrogenase and malate dehydrogenase activities were

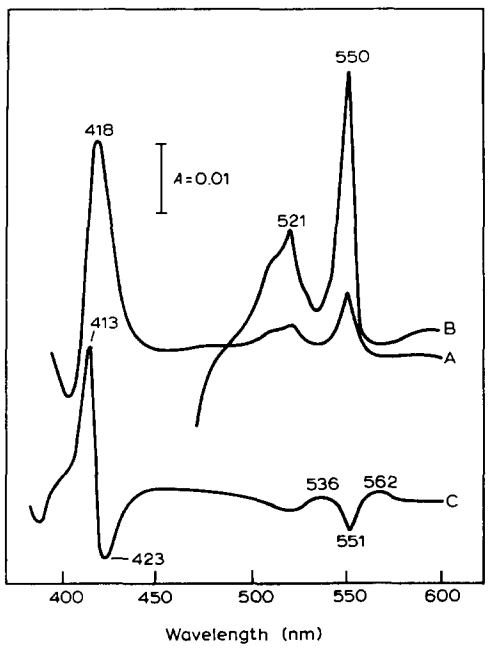

Fig. 2. Difference spectrum of the periplasmic supernatant (P). A, dithionite-reduced minus air-oxidized; B, dithionite-reduced minus ferricyanide-oxidized; C, CO difference spectrum. Protein: 1.8 mg/ml (A, B); 2.4 mg/ml (C).

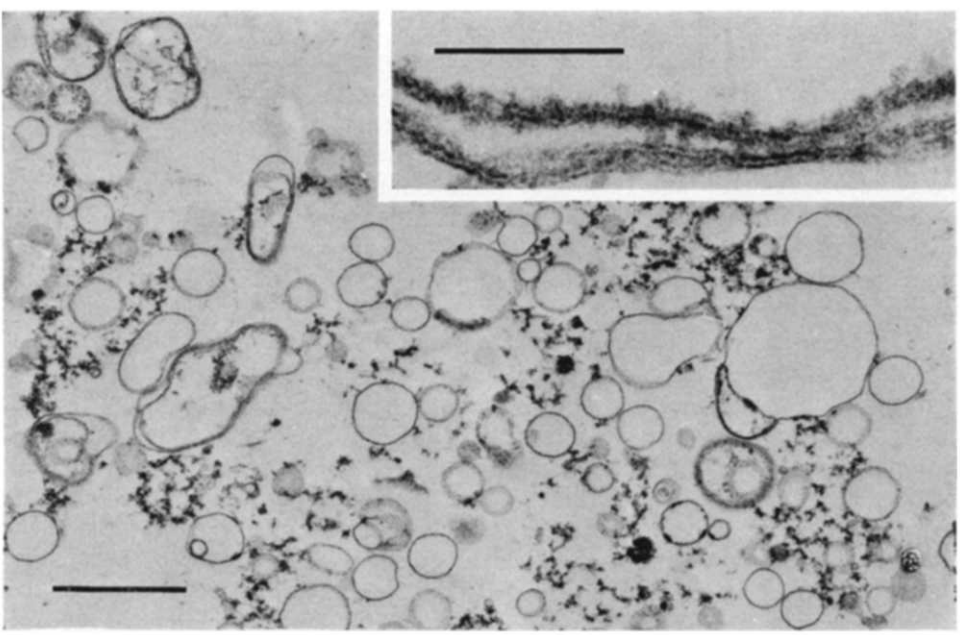

Fig. 3. Electron micrograph of a thin section of lysozyme membranes stained with uranyl acetate and lead citrate. The bars represent 1 and 0.1 μm (inset).

detected in the periplasmic supernatant; however, 19 % of the total cell cytochrome *c* was located in this fraction. The cytochrome remained soluble even after a centrifugation at 250 000 × *g* for 3 h, thus excluding contamination of the periplasmic supernatant with particulate material. The soluble fraction, containing the cytoplasmic components, retained 5 % of the total cytochrome *c* (as *c*-550), and 73 % of the total cytochrome *c* was membrane bound (*c*-553).

Morphology of the membrane fraction

Electron micrographs of thin sections of lysozyme membranes (Fig. 3) showed closed vesicles of greatly varying size (100–600 nm diameter). Large forms having the dimensions of a cell (1–1.5 μm diameter) were surrounded by two membranes, and are probably "ghosts" that had not been disrupted into small vesicles by the osmotic shock; the membrane facing the outside might be considered outer membrane and the interior one cytoplasmic membrane, respectively. Particles with about 9–10 nm diameter were attached to the membrane of some of the vesicles (Fig. 3, inset). They are similar to those found on the inner membrane of mitochondria [22, 23]; no attempt was made to determine whether the particles on the *A. eutrophus* membranes had ATPase activity. Micrographs of French press membranes showed a more homogeneous picture. The vesicles varied little in size (80–180 nm diameter, no figure), thus demonstrating the effect of harsh disruption methods.

Cytochrome and quinone content of the membranes

A. eutrophus contains membrane-bound cytochrome aa_3, *b*-560, *c*-553 and cytochrome *o* as evidenced by room-temperature difference spectroscopy [3, 4]. Difference spectra at −196 °C, however, not only separated the α-band of the cytochrome *b* from that of cytochrome *c* but distinct shoulders of the α-peak at 551 and 553 nm (Fig. 4) also suggested the presence of at least two further cytochromes. At low temperature, the absorption maxima of cytochrome *b*-560 and *c*-553 were shifted 3 nm towards the blue. The exact number of cytochromes in *A. eutrophus* has yet to be evaluated by potentiometric measurements and fourth order finite difference analysis [24].

The quantities of electron transport carriers are presented in Table II. Membrane fractions prepared from cells disrupted in the French pressure cell contained 10% less carriers than lysozyme membrane fractions; this difference was considered to be due to a different sedimentation behaviour during cell fractionation procedures. Membranes from cells cultured and harvested as described exhibited a higher content of cytochromes *b* and *c* than those described by Jones et al. [6]. In stationary phase cells, which actively stored poly-β-hydroxybutyrate (harvested at $A = 24$), a cytochrome $a_2 = d$ was found in addition to cytochrome aa_3 (no figure). The difference spectrum of these membranes showed an additional α-band at 625 nm (622 nm at −196 °C) and the pyridine haemochrome of haem a_2 had an α-band at 630 nm.

Both ubiquinone and menaquinone were extracted from lysozyme membranes [4]. Menaquinones could not be determined spectrophotometrically in the extract prepared as described by Kröger et al. [19] because of abundant quantities of ubiquinone. The determination of menaquinone was possible only after separation of both quinones by thin-layer chromatography.

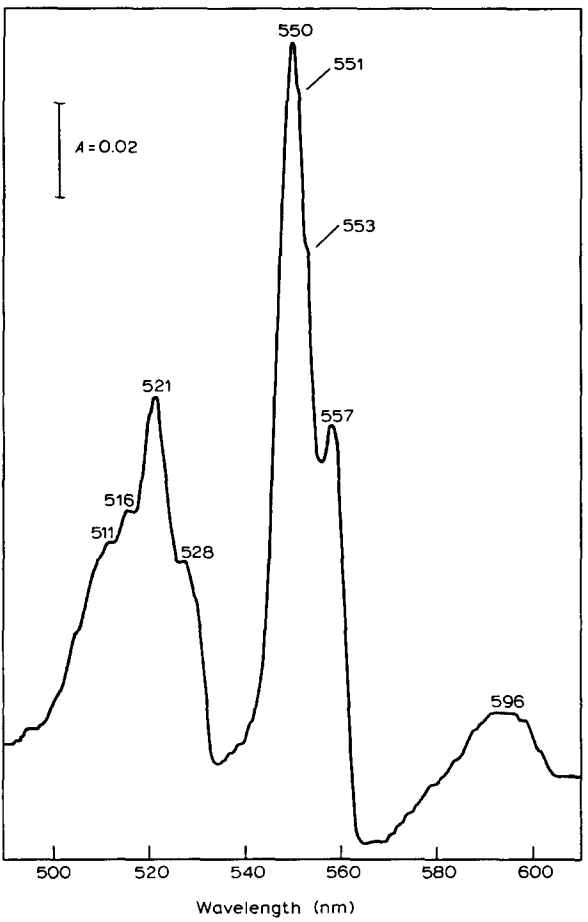

Fig. 4. Low temperature difference spectrum of lysozyme membranes. Dithionite-reduced minus oxidized spectrum. The membranes were suspended in 50 mM potassium phosphate (pH 7.0, 50 % glycerol); protein: 0.57 mg/ml.

TABLE II

CONTENT OF ELECTRON TRANSPORT COMPONENTS IN MEMBRANES PREPARED BY THE LYSOZYME METHOD

Chemical reduction was used to determine the total content of cytochromes ($Na_2S_2O_4$) and quinones (KBH_4). Values given represent the average of two separate preparations.

Component	Concentration (μmol/g protein)
Cytochrome aa_3	0.19
Cytochrome b*	0.75
Cytochrome o	0.10
Cytochrome c	1.25
Ubiquinone	2.60
Menaquinone	0.04

* Total cytochrome b content, not corrected for cytochrome o.

Membrane-bound respiratory activities

The membrane-bound respiratory activities were greatly influenced by the kind of method used for membrane preparation (Table III). All oxidase activities examined were higher in lysozyme membranes than in French press membranes; the hydrogen oxidase and the particulate hydrogenase activities were the most dramatically affected. Succinate oxidase reached maximum activity 24 h after membranes had been prepared; however, within another 24 h, the activity decreased to a stable basal value. Similarly, the NADH oxidase activity decreased to a stable value shortly after preparing the membranes. In addition to the known membrane-bound respiratory activities of *A. eutrophus* [4, 6], membrane-bound formate dehydrogenase (dichlorophenolindophenol as electron accepter) and formate oxidase activities were found. In the absence of substrate no oxygen uptake occurred, but oxygen consumption did take place if 1 mM NAD was present (the NAD solution contained no NADH as checked enzymatically by fluorimetric techniques). This kind of oxygen uptake might be interpreted as NAD-linked endogenous respiration. None of the oxidase activities examined were enhanced by the addition of soluble fraction or periplasmic supernatant to the lysozyme membranes.

To determine whether the respiratory chain activities are sensitive to an uncoupling agent, CCCP (carbonylcyanide *m*-chlorophenylhydrazone) was used to stimulate oxidase activities (Table IV). All activities examined, with the exception of the H_2 oxidase, were stimulated by CCCP to varying degrees. Stimulation of oxygen uptake was at its highest with NADPH, formate- and NAD-linked endogenous substrate (80–100 % activity increase). The NADH and succinate oxidase activities

TABLE III

EFFECT OF PREPARATION METHOD ON MEMBRANE-BOUND RESPIRATORY ACTIVITIES

Freshly harvested cells were disrupted either by lysozyme treatment or passage through a French pressure cell. Oxidase activities of the membranes were assayed polarographically (50 mM morpholinopropanesulphonic acid (pH 7.2) 10 mM $MgCl_2$, 25 °C). The substrates had the following final concentrations: succinate and formate, 10 mM; NAD, NADH and NADPH, 1 mM. H_2-oxidase activity was determined using a buffer equilibrated with a mixture of hydrogen and oxygen (85 : 15, v/v). Particulate hydrogenase activity was measured manometrically at 30 °C, as described under Methods. Values given represent the average of three different preparations.

Preparation method	Specific activity						
	Oxidase (nmol O_2/min per mg protein)					Endogenous respiration (NAD)	Hydrogenase (particulate) (nmol H_2/min per mg)
	H_2	NADH	NADPH	Succinate	Formate		
Lysozyme	330	270[a] 50[b]	42	140[a] 60[b]	50	16	1900
French pressure cell	105	220[a]	15	110[a]	23	5	660

[a] Maximal activity.
[b] Stable basal activity.

TABLE IV

STIMULATION OF MEMBRANE-BOUND OXIDASE ACTIVITIES BY THE UNCOUPLER CCCP

Test conditions are the same as given in Table III. CCCP in dimethylsulfoxide was added in μl amounts. Dimethylsulfoxide had no effect on the respiration rates. The two values of each activity reflect two different preparations.

Oxidase	Activity (nmol O_2/min)			Stimulation (%)
	−CCCP	+CCCP	(μM)	
NADH*	22.1	27.0	(200)	23.1
	30.7	39.4	(280)	28.4
NADPH	19.4	43.3	(120)	112.3
	56.7	98.5	(160)	74.0
Succinate*	51.0	57.0	(40)	12.0
	26.4	31.4	(240)	19.0
Formate	65.2	119.0	(120)	82.4
	56.7	96.5	(120)	62.3
Endogenous respiration	22.6	45.8	(120)	95.0
(NAD)	81.0	146.0	(120)	80.3

* The oxidase possessed its stable basal activity.

could only be enhanced up to 30 and 20 %, respectively, when the oxidases possessed their lower basal activities. Although the H_2 oxidase activity is stimulated by CCCP in intact cells [25], this effect could not be observed with membranes; on the contrary, CCCP ($> 10\ \mu$M) inhibited the activity. The stimulatory effect of CCCP was poor with membranes prepared from cells broken in the French pressure cell. Only the NAD-linked endogenous respiration and the formate oxidase activity were enhanced by CCCP (200 μM) up to 45 and 20 %, respectively.

Characterization of the cytoplasmic haemoprotein

When the soluble fraction was examined spectrophotometrically, the presence of a haemoprotein was discovered, which was different from the periplasmic cytochrome *c*-550, traces of which were found in this fraction. Further identification of the new component necessitated the elimination of this contaminating *c*-550; separation was achieved by column chromatography on DEAE-Sephadex as described in Methods. Cytochrome *c*-550 was washed from the column by 20 mM potassium phosphate (pH 7.0, no KCl), indicating either a neutral or alkaline isoelectric point of this pigment. The absolute spectra of the DEAE-column eluate containing the cytoplasmic haemoprotein are shown in Fig. 5. The oxidized cytochrome possessed a broad absorption maximum at 395 nm, the addition of ferricyanide or $(NH_4)_2S_2O_8$ produced no spectral changes (curve A). The dithionite-reduced cytochrome had a Soret band at 436 nm and a broad α-band at 560 nm (curve B). The reduced haemoprotein formed a CO complex with bands at 423.5, 539 and 568 nm (curve C). Thus, this haemoprotein is the major contributor to the Soret band at 420 nm in CO difference spectra of intact cells (Fig. 5, curve D). This indicates that the haemoprotein is not a *P*-450 which has been converted to a *P*-420 analogue by the cell fractionation.

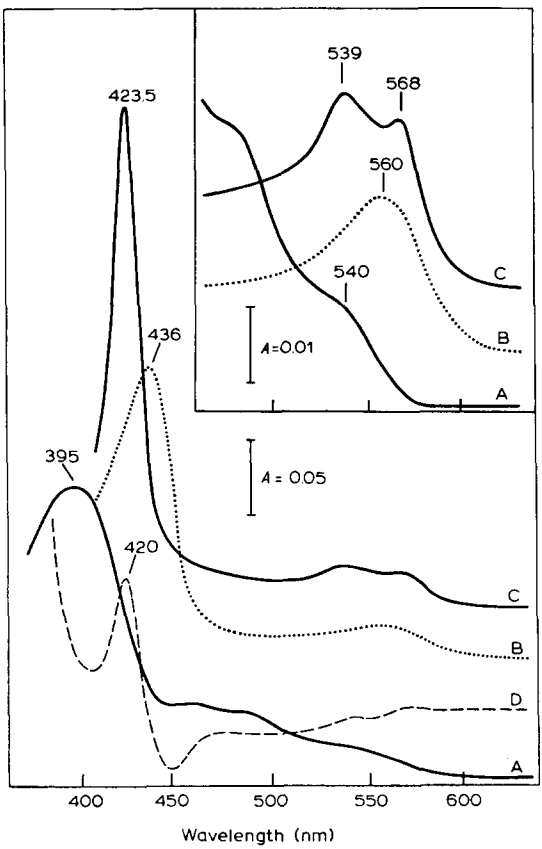

Fig. 5. Absolute spectra of the soluble haemoprotein. DEAE-column eluate: 1.6 mg protein/ml 20 mM potassium phosphate (pH 7.0). A, untreated solution; B, dithionite-reduced; C, solution of curve B bubbled 1 min with CO; D, CO difference spectrum of intact cells (2 mg protein/ml, the bar represents 0.01 absorbance units for this curve).

Reduced minus oxidized difference spectra of the haemoprotein had absorption bands at 437 and 564 nm (Fig. 6, curve A). The nature of the haem group was identified by acid acetone extraction of lyophilized DEAE column eluate (25 mg protein) and by formation of pyridine haemochromes. The haem could be separated from the protein by this procedure; the pyridine haemochrome difference spectrum of the extract showed the characteristic absorption maxima of protohaem (418, 525 and 556 nm, not shown).

As with the cytochromes *c'* [26, 27], the spectral properties of the haemoprotein were found to be dependent on pH (Fig. 6). In an alkaline solution, the absorption maxima of the dithionite-reduced haemoprotein changed to the haemochrome type with peaks at 423, 526 and 556 nm (same in absolute as well as in difference spectra), accompanied by intensification of the α-band. The transition began at alkalinities higher than pH 9 with a decrease in absorption (Fig. 6, curve B). At pH 11, the difference spectrum exhibited an intermediate form with spectral characteristics of both types (curve C); the transition was complete in 1 M NaOH (curve E). On

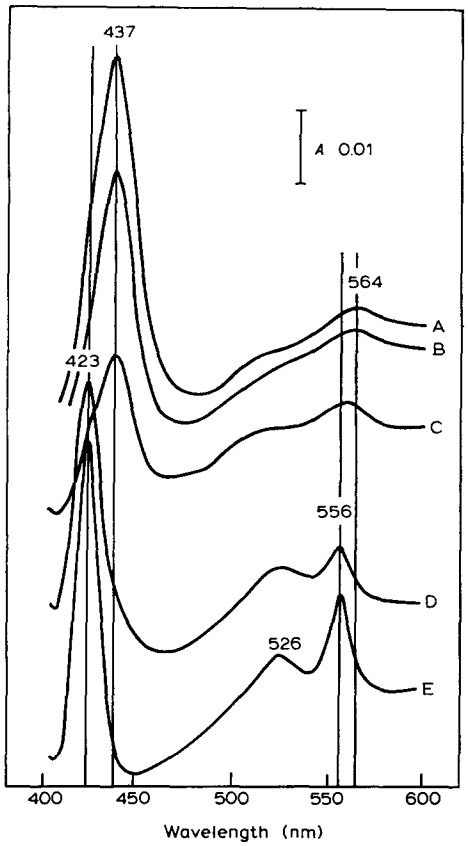

Fig. 6. Effect of pH on the difference spectrum of the haemoprotein. DEAE column eluate: 0.25 mg protein/ml 50 mM triethanolamine. Dithionite-reduced minus oxidized difference spectra: A, pH 9; B, pH 10; C, pH 11; D, 0.1 M NaOH; E, 1 M NaOH.

neutralization of the alkaline solutions, the haemoprotein regained its original difference spectrum; however, on neutralizing the 1 M NaOH, the pigment was partially denatured. The addition of NADH to aerobic solutions of the haemoprotein elicited the spectral changes observed in Fig. 7. New absorption maxima appeared at 418, 545 and 577 nm in the NADH-reduced minus untreated difference spectrum, and they increased in intensity with time. When NADH was added to the haemoprotein fraction directly after the purification procedure, the observed absorption maxima attained maximum intensity immediately, i.e. less than 1 min after the addition of NADH. After 3 weeks of storage at −20 °C, the rate of intensity increase was considerably less, as shown in Fig. 7; this suggests that one or several enzymic activities are responsible for the reduction of the haemoprotein by NADH. About 3 h after the addition of NADH, the NADH-elicited absorption maxima was replaced within 15 min by the maxima characteristic of the dithionite-reduced form of the haemoprotein (Fig. 7, curve E). The reintroduction of oxygen by inverting the cuvette several times restored the NADH-elicited

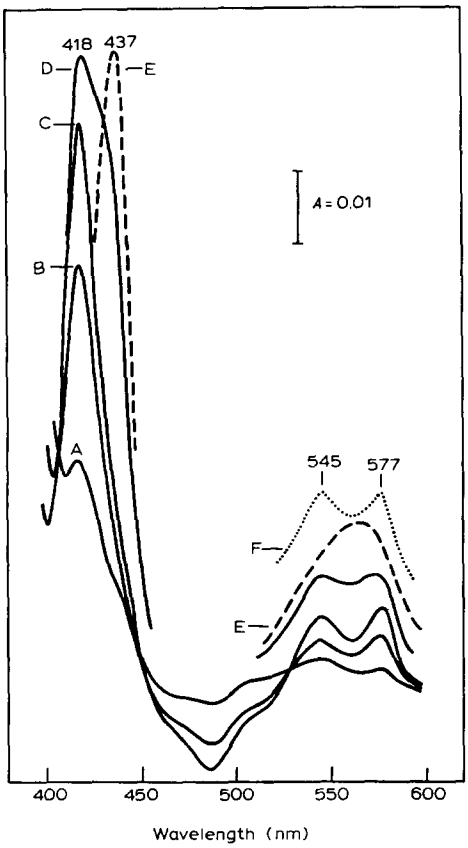

Fig. 7. Spectral changes observed as function of time after addition of NADH to the haemoprotein. DEAE-column eluate: 0.61 mg protein/ml 50 mM potassium phosphate (pH 7.0); 2 mM NADH was added. Curves A, B, C, and D, 2, 15, 25, and 180 min, respectively, after addition of NADH to the cuvette; curve E, 15 min after recording of spectrum D; curve F, cuvette inverted 20 times to admit air.

difference spectrum (curve F); addition of dithionite immediately restored the original spectrum (Soret band at 437 nm). Therefore, the form of the haemoprotein with the Soret band at 418 nm seems to require NADH and oxygen, which suggests that it is the oxygenated form of the reduced pigment. The dithionite-induced difference spectrum (Soret band at 437 nm) apparently represents the reduced non-oxygenated form. Therefore, oxygen uptake by the DEAE-column eluate with NADH as electron donor was examined polarographically to see whether the haemoprotein catalyses a reduction of oxygen. The rate of NADH-induced oxygen consumption was low, 1.47 nmol oxygen/min per mg protein (100 mM potassium phosphate (pH 7.0), 4 mM NADH, 30 °C), but comparable with the values reported by Webster and Liu [28] for the NADH-cytochrome *o* reductase from *Vitreoscilla*. Based upon the observed characteristics, the *Alcaligenes*-soluble haemoprotein bears a striking resemblance to the cytochrome *o* from *Vitreoscilla* [28, 29]. Neither guaiacol nor NAD^+ peroxidase activity was associated with the haemoprotein.

DISCUSSION

This investigation presents results on cell fractions of *A. eutrophus* H 16 obtained by applying lysozyme treatment and osmotic shock. Three distinct fractions are obtained: periplasma, membranes and cytoplasma. The advantage of a gentle procedure for the lysis of *Alcaligenes* cells over mechanic cell disruption methods, is clearly indicated by the following three results: (a) lysozyme-prepared membranes exhibit considerably higher respiratory activities than membranes prepared from cells broken in the French pressure cell, (b) membrane-bound oxidase activities are greatly stimulated by the uncoupler CCCP; this might be interpreted as a respiratory control effect which indicates the functional integrity of the membrane structure, (c) the ATPase is not solubilized but remains membrane bound.

In addition to the well-known respiratory activities in *A. eutrophus* (H_2, NAD (P)H, succinate oxidase activities; refs. 4 and 6) a membrane-bound formate oxidase activity was demonstrated. Experiments to grow the bacterium on formate failed, although CO_2 production from formate by intact cells is shown manometrically. However, in the presence of hydrogen growth on formate occurs, thus indicating that formate is able to replace CO_2 in autotrophic growth (Andreesen, M., personal communications). In this respect *A. eutrophus* is different from another hydrogen bacterium, *Hydrogenomonas* Z-1. This strain possesses an NAD-specific formate dehydrogenase which enables it to grow on formate as sole carbon and energy source [30].

Spectrophotometric investigation of the cell fractions indicated the presence of haemoproteins that had not been described before. A *c*-type cytochrome(s), *c*-550, was found in the periplasmic supernatant. It is probably leached from the outer side of the cytoplasmic membrane during the removal of the cell wall to form spheroplasts. Pyridine haemochrome spectra of the acid acetone extract show this fraction to be essentially free from *b*-type cytochromes. The weak absorption of the *c*-550 · CO complex might result from a structural alteration of the protein conformation caused by the solubilization of the pigment from the cytoplasmic membrane. This effect has been demonstrated with *Chromatium c*-type cytochromes which combine with CO only when solubilized [31]. CO difference spectra of intact cells gave no evidence of a cytochrome *c* · CO complex. However, an identification might have been obscured by the Soret band of the soluble haemoprotein. Recently, several cytochromes *c* could be localized in the periplasmic space [32, 33], as has been done with *Escherichia coli* cytochrome *c*-552 [34]. However, with the exception of the latter [35], no function could be assigned to these cytochromes.

The quantitative cytochrome content of the lysozyme membranes agrees well with the values reported previously [4], though the high value for menaquinone (0.59 μmol/g protein) found by Pfitzner [4], was not confirmed. Only trace amounts of menaquinone were found (0.04 μmol/g protein); this questions the physiological significance of menaquinone in cells which were cultured and harvested under the conditions described above. Spectra of the cytoplasmic fraction of *Alcaligenes* indicate the presence of a haemoprotein with rather broad absorption bands at 436 and 560 nm. The spectra are different from those exhibited by low spin cytochromes, but strongly resemble the spectra of peroxidases and of the cytochromes *c'* of photosynthetic bacteria. As with the cytochromes *c'*, the spectrum is dependent on pH.

Alkaline solutions transform the peroxidase-like spectrum into the typical three-banded low spin cytochrome spectrum with bands indicative of photohaem (423, 526, 556 nm). Acid acetone extraction show the haem to be non-covalently bound to the protein. The pigment is easily autoxidizable and readily combines with CO (423.5, 539, 568 nm). These characteristics suggest the protein may be a *b*-type analogue of cytochrome *c'*, as proposed by Kamen and Horio [14] for cytochrome *b*-558 from *Acetobacter suboxydans* [36] and *P*-450 from *Rhizobium japonicum* bacteroids [37]. The pigment would also fit into the flexible class of the cytochromes *o* and is similar to the cytochrome *o* purified from *Vitreoscilla* by Webster and Liu [28, 29]. This cytochrome forms an oxygenated complex when reduced with NADH in the presence of oxygen, the oxygenated complex elicits new absorption maxima at 420, 544 and 577 nm in the NADH-reduced minus untreated difference spectrum. The results presented here strongly favour an oxygenated complex of the NADH-reduced *b*-type cytochrome which elicits new bands at 418, 545, 577 nm (difference spectrum). Oxygenated complexes of reduced haemoproteins have been found in a terminal oxidase (cytochrome aa_3; [38, 39]) as well as in a hydroxylase ($P\text{-}450_{cam}$ [40]). Obviously a great deal of work is required before the function and physiological role of the soluble *b*-type cytochrome can be fully evaluated.

ACKNOWLEDGEMENTS

The electronmicroscopic work was carried out in the Department of Micromorphology, Göttingen; we wish to express our gratitude to Professor F. Mayer for his generous help. We thank Dr. D. Kuschmitz, Institut für Ernährungsphysiologie, Dortmund for the measurement of the low temperature difference spectrum. Thanks are due to Dr. J. Macy for criticism of the manuscript and helpful discussions.

REFERENCES

1 Ishaque, M., Donawa, A. and Aleem, M. I. H. (1973) Arch. Biochem. Biophys. 159, 570–579
2 Lester, R. L. and Crane, F. L. (1959) J. Biol. Chem. 234, 2169–2175
3 Ishaque, M. and Aleem, M. I. H. (1970) Biochim. Biophys. Acta 223, 388–397
4 Pfitzner, J. (1972) Zbl. Bakt. Hyg. I Abt. Orig. A 220, 396–401
5 Beatrice, M. C. and Chapell, J. B. (1974) Biochem. Soc. Trans. 2, 151–153
6 Jones, C. W., Brice, J. M., Downs, A. J. and Drozd, J. W. (1975) Eur. J. Biochem. 52, 265–271
7 Bongers, L. (1967) J. Bacteriol. 93, 1615–1623
8 Schlegel, H. G., Kaltwasser, H. and Gottschalk, G. (1961) Arch. Microbiol. 39, 209–222
9 Behn, W. and Arnold, C. G. (1974) Protoplasma 82, 77–89
10 Reynolds, E. S. (1963) J. Cell Biol. 17, 208–212
11 Taussky, H. H. and Shorr, E. (1953) J. Biol. Chem. 202, 675–685
12 Püttner, J. (1974) in Methoden der enzymatischen Analyse (Bergmeyer, H. U., ed.), Vol. 1, pp. 725–732, Chemie, Weinheim
13 Bonner, Jr., W. D. (1961) in Haematin Enzymes Symposium, Canberra, Australia (1959) (Falk, G. E., Lemberg, R. and Morton, R. K., eds.), Vol. 2, p. 479, Pergamon, Oxford
14 Kamen, M. D. and Horio, T. (1970) Annu. Rev. Biochem. 39, 673–700
15 van Gelder, B. F. (1966) Biochim. Biophys. Acta 118, 36–46
16 Chance, B. and Williams, G. R. (1956) Adv. Enzymol. 17, 65–134
17 Daniel, R. H. (1970) Biochim. Biophys. Acta 216, 328–341
18 Weston, J. A. and Knowles, C. J. (1973) Biochim. Biophys. Acta 305, 11–19
19 Kröger, A., Dadak, V., Klingenberg, M. and Diemer, F. (1971) Eur. J. Biochem. 21, 322–333
20 Schmidt, K., Liaaen-Jensen, S. and Schlegel, H. G. (1963) Arch. Microbiol. 46, 117–126

21 Kaback, H. R. (1971) in Methods of Enzymology (Jacoby, W. B., ed.), Vol. 22, pp. 99–120, Academic Press, New York
22 Fernandez-Moran, H., Oda, T., Blair, P. V. and Green, E. D. (1964) J. Cell Biol. 22, 63–100
23 Kagawa, Y. and Racker, E. (1966) J. Biol. Chem. 241, 2475–2482
24 Shipp, W. S. (1972) Arch. Biochem. Biophys. 150, 482–488
25 Hippe, H. (1967) Arch. Microbiol. 56, 248–277
26 Imai, Y., Imai, K., Sato, R. and Horio, T. (1969) J. Biochem. Tokyo 65, 225–237
27 Cusanovich, M. A., Tedro, S. M. and Kamen, M. D. (1970) Arch. Biochem. Biophys. 141, 557–570
28 Webster, D. A. and Liu, C. Y. (1974) J. Biol. Chem. 249, 4257–4260
29 Liu, C. Y. and Webster, D. A. (1974) J. Biol. Chem. 249, 4261–4266
30 Namsarev, B. B., Nozhevnikov, A. N. and Zavarzin, G. A. (1971) Mikrobiologiya 40, 772–776
31 Cusanovich, M. A. and Kamen, M. D. (1968) Biochim. Biophys. Acta 153, 376–396
32 Knowles, C. J., Calcott, P. H. and Mac Leod, R. A. (1974) FEBS Lett. 49, 78–83
33 Prince, R. C., Baccharini-Melandri, A., Hauska, G. A., Melandri, B. A. and Crofts, A. R. (1975) Biochim. Biophys. Acta 387, 212–227
34 Fujita, T. and Sato, R. (1966) J. Biochem. Tokyo 60, 568–577
35 Fujita, T. and Sato, R. (1967) J. Biochem. Tokyo 62, 230–238
36 Iwasaki, H. (1966) Plant Cell Physiol. 7, 199
37 Appleby, C. A. (1968) in Structure and Function of Cytochromes (Okunuki, K., Kamen, M. D. and Sekuzu, I., eds.), pp. 666–680, University Tokyo Press, Tokyo
38 Okunuki, K., Hagihara, B., Sekuzu, I. and Horio, T. (1958) Proc. Int. Symp. Enzyme Chemistry, Tokyo and Kyoto (1957), pp. 264–272, Pergamon Press, London
39 Lemberg, R. and Gilmour, M. W. (1967) Biochim. Biophys. Acta 143, 500–517
40 Ishimura, Y., Ullrich, V. and Petersen, J. A. (1971) Biochem. Biophys. Res. Commun. 42, 140–146