BBA 45817

PROPERTIES OF ELECTRON TRANSPORT PARTICLES FROM *HALOBACTERIUM CUTIRUBRUM*. THE RESPIRATORY CHAIN SYSTEM

K. S. CHEAH*

Department of Biochemistry, McMaster University, Hamilton, Ontario (Canada)

(Received January 30th, 1969)

SUMMARY

1. The electron transport particles (Type II) of *Halobacterium cutirubrum* were prepared by sedimentation at 79000 × *g* and by treatment with 70 mM $MgCl_2$–100 mM Tris–HCl (pH 7.6). The negatively stained sample of the electron transport particles (Type II) showed vesicles of two sizes, 330 Å and 165 Å.

2. Spectrophotometric investigations at 22° and −196° showed that the electron transport particles (Type II) contained *a*-, *b*- and *c*-type cytochromes with cytochrome *o* and cytochrome a_3 possibly acting as oxidases. The cytochrome *o* content was about two and a half times that of cytochrome a_3. Both cytochromes could be reduced by ascorbate. The *c*-type cytochrome could be reduced by ascorbate–tetramethyl-*p*-phenylenediamine (TMPD) but not by ascorbate alone.

3. Polarographic studies showed that azide and cyanide blocked ascorbate–TMPD oxidase activity and that CO inhibited O_2 uptake.

4. The extracted *o*-type cytochrome could not be reduced by ascorbate or reoxidize reduced cytochrome *c*. Its dithionite-reduced *minus* oxidized spectrum showed maxima at 559, 525 and 428 mμ; its reduced pyridine hemochromogen spectrum showed maxima at 555, 524 and 418 mμ. Thus cytochrome *o* appears to be a *b*-type cytochrome.

INTRODUCTION

Halobacterium cutirubrum, an extreme halophile, can grow only in a medium containing a very high salt concentration[1]. This obligate, aerobic bacterium requires at least 15% NaCl in the culture medium for growth[2], which is optimal between 25 and 30% NaCl. Below this critical value growth ceases; and, at decreasing salt concentrations, the bacterium transforms its rod-shaped structure to spheres. Ultimately lysis occurs when the salt content is less than 5–10%.

Various enzymes were demonstrated in the cell-free extracts of the extreme halophile, *Halobacterium salinarium*[3], and NADH oxidase activity[4] was observed in *H. salinarium*, *H. cutirubrum* and *Halobacterium halobium*. Except for the report of cytochrome oxidase activity[5], practically no reference to either cytochrome partici-

Abbreviations: HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; TMPD, tetramethyl-*p*-phenylenediamine.

* Present address: Biochemistry Department, McGill University, Montreal, Canada.

pation or to the pathway of electron transport has yet been reported in these extreme halophiles. This paper reports, firstly, the preparation and properties of the "electron transport particles" of *H. cutirubrum* and, secondly, the extraction of an *o*-type cytochrome from the precipitate originally sedimented at 79000 × *g*.

MATERIALS AND METHODS

Reagents

Antimycin A (Type III), L-ascorbate, DL-α-glycerophosphate (disodium salt, grade X) and Tris were obtained from the Sigma Corp.; tetramethyl-*p*-phenylenediamine (TMPD) from the British Drug Houses; and all other reagents were of analytical grade. CO was purchased from the Matheson Corp. Deionized, distilled water was used throughout.

Preparation of electron transport particles from H. cutirubrum

The red-pigmented bacterium, *H. cutirubrum*, was grown aerobically, harvested during its logarithmic growth phase, washed, and homogenized as previously described by BAYLEY AND GRIFFITHS[6]. The homogenate was centrifuged at 54000 × *g* for 20 min at 0° in a Beckman (Model L2 65B) ultracentrifuge using a 65 rotor. The supernatant was then centrifuged at 79000 × *g* for 30 min thus giving a clear supernatant, designated as 79 S, and a red pellet. The pellets from the two centrifugations were homogenized (5–10 passes of the pestle) separately with 2–3 vol. of 100 mM Tris–HCl buffer (pH 7.6) containing 70 mM $MgCl_2$ in an all glass Potter–Elvehjem homogenizer in an icebath. Furthermore, both suspensions were diluted separately with the homogenizing medium (20–25-fold for the 54000 × *g* pellet, 5–10-fold for the 79000 × *g* pellet) and after standing in an icebath at 4° for 18 h were separated into a brick-red precipitate and an orange-red, turbid supernatant. The precipitate derived from the 54000 × *g* and 79000 × *g* pellet are designated as "Type I" and "Type II electron transport particles", respectively.

Separation of the o-type cytochrome

The orange-red, turbid supernatant from the 79000 × *g* pellet was centrifuged at 4400 × *g* for 6 h in a Sorvall Superspeed Angle Centrifuge (Model SS-I) using a SS-34 rotor. The clear supernatant (140 ml, Fraction I) then was dialysed for 30 h against five changes of deionized, distilled water. The clear dialysate was concentrated 5.7-fold by layering a total of 25 g of dry Sephadex G-200, in 5-g portions, on the dialysis tube. The concentrated dialysate was centrifuged at 4400 × *g* for 1 h, and the clear supernatant (25 ml) was deep frozen at −20° in 5.0-ml portions (Fraction II). 5.0 ml (Fraction II) were applied to a Sephadex G-200 (40–120 μ) column (1.5 cm × 72 cm) previously equilibrated with 0.05 M phosphate buffer at pH 7.4 and at 4°. Elution was carried out with 0.05 M phosphate buffer (pH 7.4). 2.5-ml fractions were collected at a flow rate of approx. 3.3 ml/h. The first pink band was completely eluted between the 11th and 20th fraction inclusive (Fraction III).

Extraction and identification of the prosthetic group of the o-type cytochrome

An acid methyl ethyl ketone extract of the *o*-type pigment was prepared by the method of TEALE[7] using 0.5 ml of Fraction III. The pyridine hemochromogen of the methyl ethyl ketone extract was formed by treating it with an alkaline pyridine

solution followed by 1.0 mg dithionite. The spectrum of the reduced pyridine hemochromogen of the extract was recorded using methyl ethyl ketone as the blank.

Physicochemical measurements

Respiratory activities were measured polarographically at 25° using a Clark oxygen electrode (Yellow Springs Instrument Co., Ohio) in a magnetically stirred suspension in a 3.0-ml closed vessel. Additions to the reaction vessel were made in small volumes through a capillary port with Hamilton microsyringes. The reaction medium was 70 mM $MgCl_2$–100 mM Tris–HCl (pH 7.6), and was the same medium as that used for measuring the NADH oxidase activity of the extreme halophile, *H. salinarium*[4], except that the pH was 7.6 instead of 7.4.

Ascorbate–TMPD oxidase activity, determined polarographically, was based on the method of LEE *et al.*[8] except that the reaction mixture was 70 mM $MgCl_2$–100 mM Tris–HCl (pH 7.6). The ascorbate and TMPD were added to the reaction medium in 0.01-ml additions; the sequence of addition was 0.1 mM TMPD, 1.0 μg antimycin A per mg protein, 2.2 mM ascorbate and either azide or cyanide as an inhibitor or ascorbate, antimycin A, TMPD and inhibitor.

Glucose-6-phosphatase (EC 3.1.3.9) activity was determined as described by ROODYN *et al.*[9], and protein was determined by the method of LOWRY *et al.*[10] using bovine serum albumin as standard.

The respiratory components of the electron transport particles were detected without prior extraction of the carotenoid pigments, reported to be present in this bacterium[11]. All spectra were recorded at room temperature with a Cary (Model 14) spectrophotometer fitted with a high intensity light source. Difference spectra at 77°K were recorded at the Johnson Research Foundation with a split beam spectrophotometer[12] using the "trapped steady state" technique[13,14].

The concentrations of various cytochromes in the electron transport particles were calculated from difference spectra by using the millimolar extinction coefficient of 80 for cytochrome *o* (ref. 15), of 19.1 for cytochrome *c* (ref. 16) and of 16 for cytochrome *b* (ref. 17) (α-peak at 557 mμ). Cytochromes *a* and a_3 were calculated by the method of VAZQUEZ-COLON AND KING[18]. All these calculations were arbitrary, since no coefficients were available for purified *H. cutirubrum* cytochromes.

The pyridine hemochromogens of the electron transport particles were formed by treating an equal volume of electron transport particles with an equal volume of an alkaline pyridine solution. The pyridine hemochromogens were reduced with dithionite, and a difference spectrum was recorded from 700 to 390 mμ.

For electron microscopic studies, samples of the Type I and Type II electron transport particles, which were diluted 5 times with the suspending medium, were negatively stained for 2 min with 2% phosphotungstic acid (pH 6.8) using Formvar carbon-coated grids. The excess phosphotungstic acid was removed with filter paper, and the specimen then was dried and examined with a Philips (Model EM 300) electron microscope.

RESULTS

Electron microscopy

Fig. 1 illustrates the electron micrographs of the negatively stained samples of the two types of electron transport particles prepared from *H. cutirubrum*. The

electron micrograph of Type I electron transport particles (Fig. 1A) shows a heterogeneous mixture of fragments of membranes and vesicles of various sizes; the largest is about 900 Å in diameter. Fig. 1B indicates that Type II electron transport particles

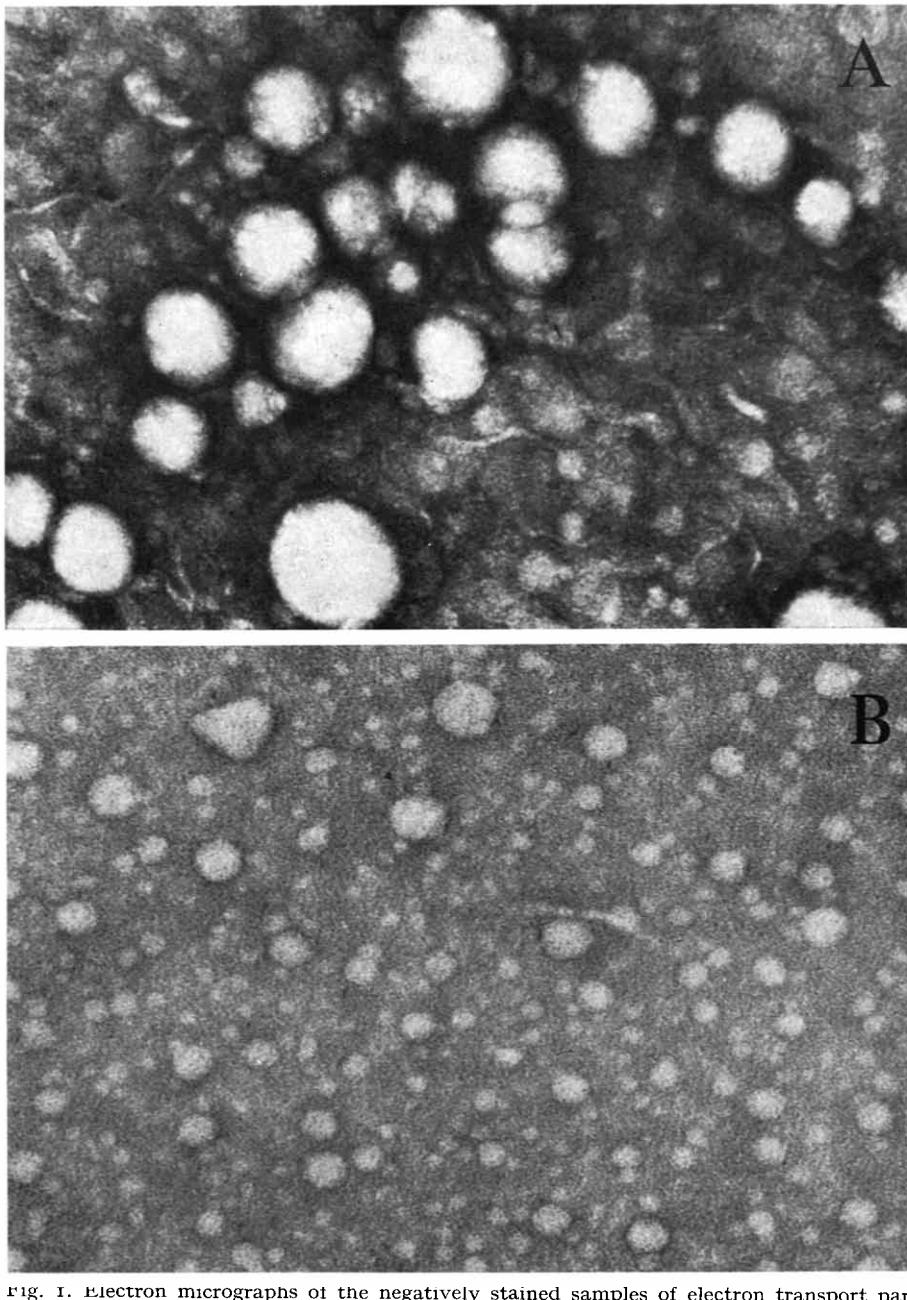


Fig. 1. Electron micrographs of the negatively stained samples of electron transport particles prepared from *H. cutirubrum*. Samples of electron transport particles stained with 2% phosphotungstic acid (pH 6.8) using Formvar carbon-coated grids. (A) Type I electron transport particles; magnification × 151 200. (B) Type II electron transport particles; magnification × 189 000.

are more homogeneous: vesicles of two sizes are observed of which the larger is about 330 Å and the smaller about 165 Å in diameter.

Properties of the electron transport particles

Investigations were conducted with the Type II electron transport particles because the preparation was more homogeneous than Type I. Type II electron transport particles could be reduced by α-glycerophosphate and ascorbate. No other substrate was tested. Fig. 2 illustrates the difference spectra (22°) recorded at 3 min following the addition of either α-glycerophosphate or ascorbate. Both substrates reduced the same *a* (606 mμ), whereas ascorbate reduced a single *b*-type pigment (α-peak) at 560 mμ and α-glycerophosphate produced a strong absorption at 557 mμ.

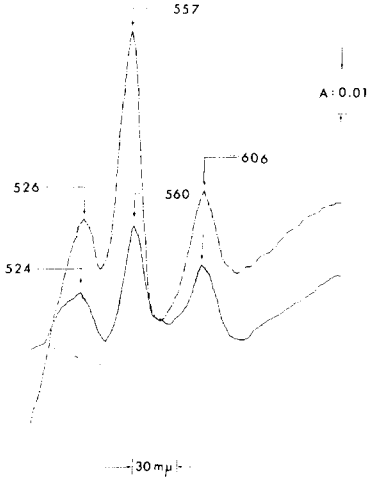


Fig. 2. Room temperature (22°) difference spectra of the electron transport particles from *H. cutirubrum*. Both the sample and reference cuvettes (5.0-mm light path) contained 1.25 ml electron transport particles (18.3 mg protein/ml) in 70 mM $MgCl_2$–100 mM Tris–HCl (pH 7.6). – – – –, baseline; –·—·–, α-glycerophosphate-reduced *minus* oxidized; ———, ascorbate-reduced *minus* oxidized. Difference spectra recorded 3.0 min after substrate addition. The reference systems were oxidized by shaking in air before scanning. Final concentrations: α-glycerophosphate, 6.0 mM; ascorbate, 3.0 mM.

α-Glycerophosphate could also have reduced the 560 mμ component, but its absorption peak probably was masked by the predominant band at 557 mμ. The dithionite-reduced sample at 22° (not shown) shows only the presence at 606 mμ of *a*-type and at 559 mμ of *b*-type cytochromes. The results suggest that the electron transport particles have more than one *b*-type pigment and that dithionite reduced an additional *b*-type cytochrome which was different from those reduced by either ascorbate or α-glycerophosphate. This was clearly shown by recording the difference spectrum of the dithionite-reduced samples against the α-glycerophosphate-treated ones. Such a spectrum shows the presence of a *b*-type cytochrome with maxima at 561 (α), 532 (β) and 431 (γ) mμ. The 79-S fraction contained neither substrate nor dithionite-reducible pigment which shows that all the cytochrome components were found in the electron transport particles.

The site of inhibition by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) on the respiratory chain system was investigated using α-glycerophosphate as substrate.

Fig. 3 shows that the addition of HQNO to the α-glycerophosphate-reduced particles followed by O_2 resulted firstly in the reoxidation of the *a*- (605, 443 mμ) and *b*- (557, 425 mμ) type cytochromes and, secondly, in the appearance of a *c*-type component with maxima at 553 and 423 mμ. This result suggests that HQNO can block the electron flow somewhere between the *c*- and *a*-type cytochrome instead of the expected site between the *b*- and the *c*-type pigment. The same concentration of inhibitor without O_2 had no effect on the reduced cytochromes in the anaerobic state.

The reduced pyridine hemochromogen difference spectrum of the electron transport particles shows α-peaks at 587 and 553 mμ. The 587 mμ band is due to heme *a* and the 553 mμ peak to that of the fused peaks of heme *c* and protoheme[19].

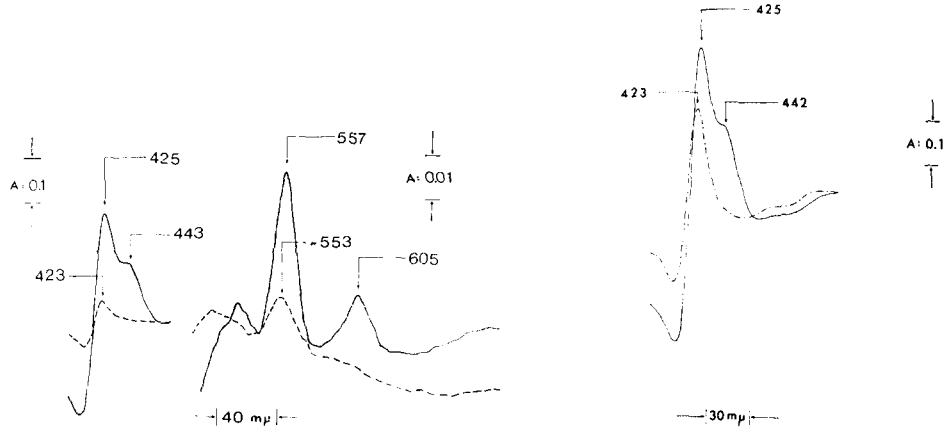


Fig. 3. Effect of HQNO on the α-glycerophosphate-reduced electron transport particles (22°). Both cells (5.0-mm light path) contained 1.25 ml electron transport paritcles (13.2 mg protein/ml). ———, α-glycerophosphate-reduced *minus* oxidized; – – – –, α-glycerophosphate-reduced + HQNO + O_2 *minus* oxidized. Final concentrations: α-glycerophosphate, 6.0 mM; HQNO, 1.5 μg/mg protein.

Fig. 4. Room temperature spectra showing the effect of ascorbate on the α-glycerophosphate-reduced spectrum. Similar procedure as described in Fig. 2. ———, α-glycerophosphate-reduced *minus* oxidized; –·–·–·–, α-glycerophosphate-reduced *minus* ascorbate-reduced.

The concentrations of heme *a*, *b* and *c* were approx. 0.05, 0.44 and 0.23 mμmole of heme per mg protein, providing that there was no decomposition of heme occurring when the reduced pyridine hemochromogens were determined directly from the electron transport particles.

Ascorbate donates electrons to the respiratory chain at a point much closer to the O_2 side than α-glycerophosphate in the electron transport particles because the *a*-type pigment at 606 mμ was detected 30 sec following ascorbate but was seen much later following α-glycerophosphate addition. Further supporting this conclusion is Fig. 4 with the results obtained from the α-glycerophosphate-reduced *minus* oxidized and the α-glycerophosphate *minus* ascorbate difference spectra. The addition of ascorbate to the reference cell caused, firstly, the disappearance of the Soret peak at 442 mμ of the *a*-type pigment and, secondly, approx. 50% collapse of the 425 mμ band, which was replaced by a symmetrical peak at 423 mμ. The 423 mμ maximum could be contributed by either a mixture of *b*- and *c*- or by a *c*-type pigment.

Fig. 5 illustrates the difference spectra of the electron transport particles at the temperature of liquid nitrogen. The dithionite-reduced spectrum (B) shows the presence of *a*- (605, 443 mμ), *b*- (558 mμ) and *c*- (549 mμ)type cytochromes of which only the *a*- and another *b*-type (560 mμ) were reduced by ascorbate (A). The 560 and 558 mμ peaks were contributed by different components whose Soret bands appeared at 425 and 430 mμ. The ascorbate-reduced spectrum confirmed the results obtained at room temperature in that ascorbate could reduce only the *a* and *b* cytochromes but not the *c*-type cytochrome in the electron transport particles.

Fig. 6 represents the difference spectra recorded at the temperature of liquid nitrogen showing both the effect of cyanide on the pigments reduced by ascorbate–TMPD and the position at which ascorbate–TMPD donates to the respiratory chain system of the electron transport particles. Ascorbate–TMPD (A) caused the reduction of the *a*-(440 mμ)type cytochrome and also of the pigments with a Soret band at 424 mμ. The subsequent addition of cyanide (B) to the ascorbate–TMPD-treated sample resulted in a 30% increase in absorbance of the 424 mμ band and in a shift of the *a*-type cytochrome peak from 440 to 437 mμ and the 460 mμ trough to 475 mμ. A pronounced shoulder at about 450 mμ also was observed in the presence of cyanide.

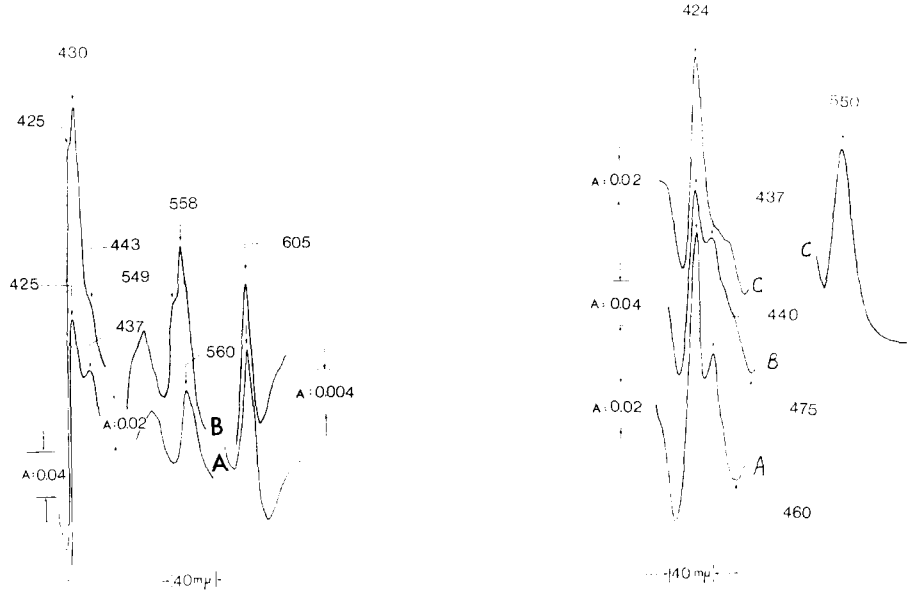


Fig. 5. Difference spectra of electron transport particles at liquid-nitrogen temperature showing the dithionite and ascorbate-reducible cytochromes. Both the reference and sample cuvettes (2.0-mm light path) contained 0.35 ml electron transport particles (5.1 mg protein/ml). The electron transport particles were reduced with 1.0 mg dithionite (B) and 3.0 mM ascorbate (A). The electron transport particles preparation used was that which had previously been frozen and stored at liquid-nitrogen temperature.

Fig. 6. Liquid-nitrogen temperature difference spectra showing the effect of cyanide and ascorbate addition to the ascorbate–TMPD-reduced pigments. Similar procedure as that described in Fig. 5. A, ascorbate–TMPD reduced *minus* oxidized; B, ascorbate–TMPD + cyanide *minus* oxidized; C, ascorbate–TMPD reduced *minus* ascorbate-reduced. Final concentrations: ascorbate–TMPD, 3.0–0.16 mM; cyanide, 1.0 mM; ascorbate, 3.0 mM.

As mentioned before (Figs. 2 and 5A), ascorbate without TMPD could reduce the *a*- and *b*-type pigments, the latter having an α-peak at 560 mμ. In order to determine the position(s) at which ascorbate and ascorbate–TMPD donate to the respiratory chain, a difference spectrum was recorded where one sample of the electron transport particles was treated with ascorbate–TMPD and the reference cell with ascorbate. The results obtained (C) show a maximum at 550 mμ and also a single peak at 424 mμ, which was about 20% less than that obtained with ascorbate–TMPD (A). The evidence shows that ascorbate alone donates to a different position of the respiratory chain than ascorbate–TMPD; the latter acts at a point further away from the O_2 side, *i.e.* at the level of the *c*-type cytochrome.

The rates of reduction of the *a*- and *b*-type cytochromes by ascorbate were followed at a fixed wavelength with the Cary spectrophotometer, the *a*-type at 443 mμ and the *b*-type at 560 mμ. As shown in Fig. 7A, ascorbate reduced the *a*-type cytochrome, and the aerobic steady state was reached at about 1 sec after the addition of ascorbate while anaerobiosis occurred at about 60 sec. Dithionite caused a further increase in the reduction. Assuming that dithionite gave a 100% reduction the *a*-type pigment(s) was reduced about 33% in the aerobic steady state and 80% on reaching anaerobiosis. With the *b*-type cytochrome, the aerobic steady state reduction was reached at about 1 sec after ascorbate addition (B) just like the *a* type pigment.

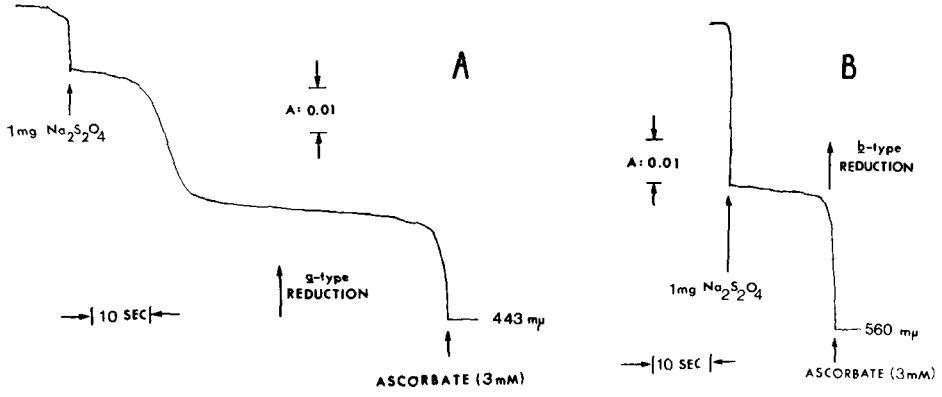


Fig. 7. Spectrophotometric tracing showing the rate of reduction of the *a*- and *b*-type cytochromes by ascorbate and the reduction by dithionite of the electron transport particles at room temperature. The rate of reduction of the *a*- and *b*-type cytochromes was followed at a fixed wavelength with a Cary (Model 14) spectrophotometer using 10 mm cells containing 2.5 ml electron transport particles (12.6 mg protein/ml).

Dithionite gave a further increase in reduction. The results show that both the *a*- and the *b*-type cytochromes were reduced at a fast rate by ascorbate.

The nature of the terminal oxidase(s) of the electron transport particles was tentatively determined by the formation of CO complex(es) in a CO difference spectrum (reduced + CO *minus* reduced spectrum). Fig. 8 represents such a spectrum obtained with ascorbate as the reducing agent. The results clearly demonstrate the formation of two types of CO complexes which suggest the presence of cytochrome a_3 and an *o*-type pigment in the electron transport particles. The 593 mμ peak corresponded to that of the cytochrome a_3–CO complex; the peaks at 578, 539 and 419 mμ and the trough at 560 mμ were contributed by the CO complex of an *o*-type

pigment. The 442 mμ trough, whose position was about 8–10 mμ longer than that attributed to an *o*-type pigment alone[20,21], probably represents the combined troughs of cytochrome a_3–CO (445 mμ) and cytochrome *o*–CO (432 mμ) complexes. The expected Soret peak of cytochrome a_3–CO (430 mμ) was not discernible but might have been obscured by the predominant cytochrome *o*–CO peak[22].

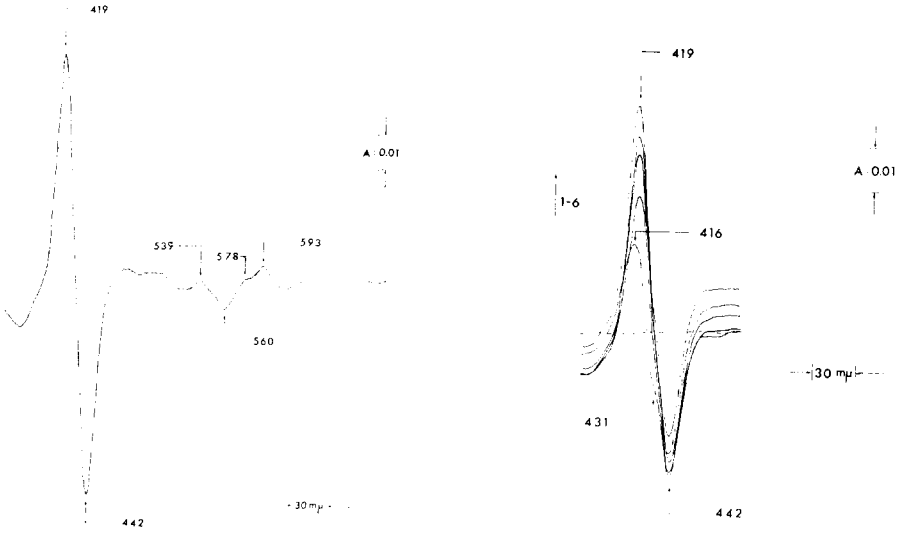


Fig. 8. CO difference spectrum (ascorbate + CO *minus* ascorbate) at room temperature of the electron transport particles from *H. cutirubrum*. Both the sample and reference cuvettes (5.0-mm light path) containing 1.25 ml electron transport particles (14.0 mg protein/ml) were reduced with ascorbate (3.0 mM). CO was bubbled through the sample cuvette after a satisfactory straight baseline was obtained.

Fig. 9. Time-dependent changes of the CO difference spectra (α-glycerophosphate + CO *minus* α-glycerophosphate) of the electron transport particles from *H. cutirubrum*. Both the sample and reference cuvettes (5.0-mm light path) containing 1.0 ml of electron transport particles (8.13 mg protein) were reduced with α-glycerophosphate (6.0 mM). CO was bubbled through the sample cuvette for 1.0 min after a satisfactory baseline was obtained. (1) ——, baseline; – – – –, CO difference spectra recorded as indicated in the direction of arrow at (2) 1.0 min; (3) 4.0 min; (4) 7.0 min; (5) 10.0 min; (6) 15.0 min after CO treatment.

However, the Soret cytochrome a_3–CO peak could be observed following brief incubation with CO of the reduced electron transport particles. This was based on the observations that CO would bind with cytochrome a_3 faster than with cytochrome *o* as reported for *Bacillus megaterium*[23] and for the parasitic worm, *Moniezia expansa*[22]. Fig. 9 represents the time-dependent changes of the CO difference spectrum (α-glycerophosphate + CO *minus* α-glycerophosphate) of the electron transport particles. The CO difference spectrum recorded at 1.0 min following CO treatment clearly shows a pronounced shoulder at 431 mμ contributed by the Soret peak of cytochrome a_3–CO complex. The 416 mμ maximum of cytochrome *o*–CO peak increased its absorbance and gradually shifted to 419 mμ with a longer period of CO incubation. This observation was similar to that observed by BROBERG AND SMITH[23] in *B. megaterium* which also has more cytochrome *o* than cytochrome a_3.

TABLE I

APPROXIMATE CONCENTRATIONS OF THE RESPIRATORY COMPONENTS IN THE ELECTRON TRANSPORT PARTICLES OF *H. cutirubrum*

All the cytochromes except cytochrome *c* were calculated from room temperature difference spectra. Cytochrome *c* concentration was roughly determined from the dithionite-reduced *minus* oxidized spectrum at liquid-nitrogen temperature using the millimolar extinction coefficient of 19.1 for the cytochrome *c* (ref. 16).

Respiratory components	*Concn. (mμmole/mg protein)*
Cytochrome b_{557}	0.47*
Cytochrome *c*	0.30
Cytochrome *a*	0.08*
Cytochrome a_3	0.11*
Cytochrome *o*	0.28**
Cytochrome a_3	0.12**

* Calculated from α-glycerophosphate *minus* oxidized spectrum (Fig. 2).
** Calculated from CO difference spectrum (ascorbate + CO *minus* ascorbate) (Fig. 8).

Table I illustrates the approximate concentrations of the respiratory components in the electron transport particles. The component (b_{557}) refers to the *b*-type pigment reduced by α-glycerophosphate which showed an α-peak at 557 mμ in the difference spectrum (Fig. 2). The concentration of this cytochrome was calculated using the millimolar extinction coefficient of cytochrome b_1 (ref. 17). The results show that the electron transport particles have more cytochrome *o* than cytochrome a_3; the cytochrome *o* was about 2.5 times that of cytochrome a_3.

Glucose-6-phosphatase activity was not detected in either the Type I or Type II electron transport particles.

Polarographic studies

The sensitivity of the terminal oxidase activity of the electron transport particles towards azide and cyanide was investigated using ascorbate–TMPD as an electron donor. The ascorbate–TMPD oxidase activity (30–32 mμmoles O_2 per min per mg protein at 25°) which was insensitive to antimycin A, was inhibited 44% by 1.0 mM azide and 84% by 0.5 mM cyanide.

The inhibition of respiration by CO was investigated using ascorbate which could reduce the two presumed terminal oxidases, cytochrome a_3 and an *o*-type pigment. The CO effect on ascorbate oxidation was determined polarographically in a CO-saturated reaction medium of 70 mM $MgCl_2$–100 mM Tris–HCl (pH 7.6) at 25°. The control contained no CO. Under such conditions, CO inhibited about 20% of the O_2 uptake.

Spectral properties of the extracted o-type pigment

Fig. 10 illustrates the spectrophotometric tracing of the CO difference spectrum of the *o*-type pigment extracted from *H. cutirubrum*. The CO difference spectrum of

Fraction I, recorded 5.0 min after the CO treatment, shows only one type of CO-binding pigment with peaks at 575, 538 and 418 mμ and corresponding troughs at 558 and 440 mμ (A). The position of the 440 mμ trough was about 7 mμ longer than that normally observed for the trough of a CO complex contributed by an *o*-type pigment in the Soret region and suggested that something else was contributing in this region such as the trough of cytochrome a_3–CO complex. The CO difference

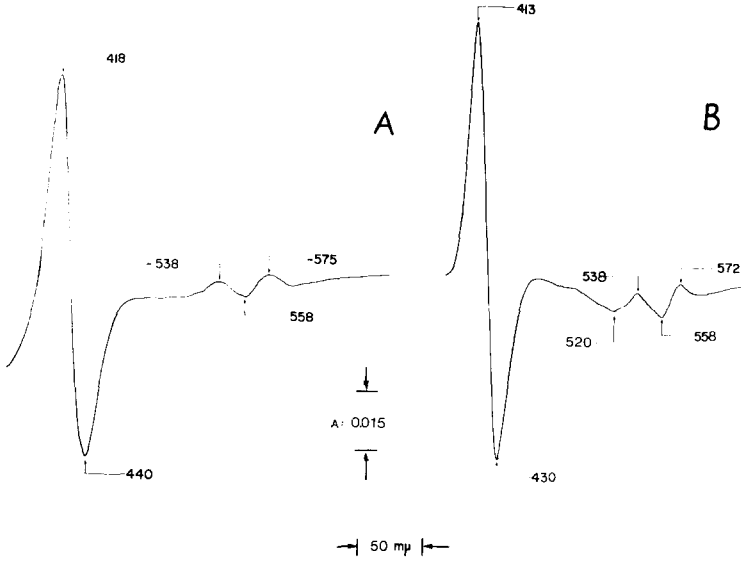


Fig. 10. CO difference spectra (dithionite + CO *minus* dithionite) of the *o*-type cytochrome in Fraction I and Fraction II. Procedure similar to that described in Fig. 8 except that 10-mm cells were used. (A) Fraction I, CO difference spectrum. (B) Fraction II, CO difference spectrum.

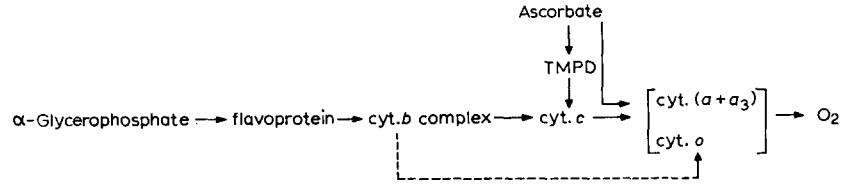
spectrum of Fraction II concentrated on Sephadex (B) showed maxima at 572, 538 and 413 mμ with corresponding minima at 558, 520 and 430 mμ. The ratio of the difference in absorbance changes between the wavelength pairs, 413–430 and 572–558 mμ, was 14.1:1. This value corresponds fairly well with the value of 15.0:1 reported for the cytochrome *o*–CO complex for the particle suspension of *Staphylococcus aureus* and 14.7:1 for intact cells of *Staphylococcus albus*[21].

The first pink band (Fraction III) eluted from the Sephadex G-200 column was concentrated using Sephadex G-200. The concentrated Fraction III shows the presence of a CO-binding pigment with absorption maxima and minima similar to those reported in Fraction II, prior to passing through Sephadex G-200. Also, the same ratio of absorbance changes between the wavelength pairs 413–430 and 572–558 mμ exists. Fraction III, on reduction with dithionite, showed absorption bands at 559, 525 and 428 mμ and a trough at 408 mμ in the difference spectrum, suggesting that the pigment is a *b*-type. The 525 mμ band appeared as a shoulder rather than as a pronounced peak.

The reduced pyridine hemochromogen spectrum of the methyl ethyl ketone extract showed peaks at 555, 524 and 418 mμ, indicating the presence of protoheme[24] and thus showing that the *o*-type pigment is a *b*-type cytochrome.

DISCUSSION

The data presented show that the electron transport particles prepared from the 79000 × *g* pellet contained *a*-, *b*- and *c*-type cytochromes with possibly two terminal oxidases. The inhibition of O_2 uptake by CO, the inhibition of the ascorbate–TMPD oxidase activity by azide and cyanide, and the formation of the CO complexes suggest that cytochrome *o* and cytochrome a_3 could possibly be the two functional oxidases in the electron transport particles prepared from *H. cutirubrum*. The following scheme illustrates the proposed basic respiratory chain system of the electron transport particles of *H. cutirubrum*.



The alternate pathway (broken line) linked cytochrome *b* (at 557 mμ) to the terminal oxidases and by-passed cytochrome *c*. The cytochrome *b* complex includes all the *b*-type pigments reduced by dithionite (α-peak at 559 mμ), α-glycerophosphate (α-peak at 557 mμ), and the component with an α-peak at 561 mμ in the dithionite *minus* α-glycerophosphate difference spectrum.

One of the most interesting features observed was that ascorbate could reduce the cytochrome *o* and cytochrome ($a + a_3$) without the participation of a *c*-type pigment. The rate of reduction of these pigments was quite rapid, with the aerobic steady-state occurring at about 1 sec following ascorbate addition. The *c*-type cytochrome, detected at the temperature of liquid nitrogen could be reduced by ascorbate–TMPD.

The electron transport particles prepared from the 79000 × *g* pellet (Type II) using 100 mM Tris–HCl (pH 7.6) containing 70 mM $MgCl_2$ appears to be quite homogeneous, and the method of preparation described is quite reproducible. Only one out of four attempts was unsuccessful in that no separation occurred after standing at 4° for 18 h in an icebath, as described in MATERIALS AND METHODS. However, when these particles did not separate on standing, a pellet could be obtained after centrifuging at 4400 × *g* for 1 h at 0°. This pellet contained the same type of electron transport particles observed by negative staining and the same pattern of respiratory components. The exact mechanism by which $MgCl_2$ *plus* Tris–HCl leads to the formation of the vesicles detected by negative staining is not known. These vesicles are unlikely to be artifacts as the negatively stained sample of the dialysed electron transport particles showed no such structures. Alternatively, the main features of the electron micrographs could also be interpreted as probably due to an excess of dissolved substances and might not necessarily represent the true morphology of the particles being investigated. In contrast, the Type I electron transport particles could be prepared reproducibly, but unfortunately the preparation was heterogeneous. Both the Type I and Type II electron transport particles have no glucose-6-phosphatase activity and also contained carotenoid pigments which are present in *H. cutirubrum*[11].

The procedure for preparing the electron transport particles (Type I and Type II)

also excluded an *o*-type cytochrome, detected in the supernatant fractions. This cytochrome from the preparation of Type II electron transport particles was purified 2.6-fold on a protein basis and had a peak at 413 mμ and a trough at 430 mμ in the CO difference spectrum with dithionite as the reducing agent. The cytochrome *o* CO complex of the electron transport particles (Type II) and the extract (Fraction I), before being concentrated with Sephadex, showed a maximum at 418 and a trough at 440 mμ. The concentrated dialysate (Fraction II) and the Sephadex eluate (Fraction III) contained an *o*-type cytochrome with the CO complex having a Soret peak at 413 and a trough at 430 mμ. It is difficult to explain the discrepancies observed. It is quite possible that the extracted cytochrome *o* could be an isolation artifact or that cytochrome a_3 was removed and/or destroyed. The pigment could not be reduced by ascorbate or reoxidize reduced cytochrome *c*.

During the preparation of this manuscript the electron transport system of *H. cutirubrum*, harvested at an early stationary phase, was reported by LANYI[25]. The major difference between the two results is that LANYI[25] failed to detect any substrate or dithionite reducible cytochrome a_3 and cytochrome *o*.

ACKNOWLEDGMENTS

The author wishes to express his thanks to the following: Dr. F. D. Ziegler for providing laboratory facilities, Dr. S. T. Bayley and Mr. B. White for constant supply of the bacterium, Dr. J. F. Mustard for use of the electron microscope, Mr. T. Bistricki for preparing the photomicrographs and Mrs. M. Wills for protein determination. The author is indebted to Dr. B. Chance of the Johnson Research Foundation, University of Pennsylvania, for allowing the use of his split-beam spectrophotometer for recording the low temperature spectra and to the Canadian Medical Research Council for a Research Fellowship (1967–1968). This project was supported in part by grant (MA 1950) from the Medical Research Council of Canada.

REFERENCES

1 H. LARSEN, in I. C. GUNSALUS AND R. Y. STANIER, *The Bacteria*, Vol. 4, Academic Press, New York, 1962, p. 297.
2 H. LARSEN, *Advan. Microbiol. Physiol.*, 1 (1967) 97.
3 P. K. HOLMES, I. E. D. DUNDAS AND H. O. HALVORSON, *J. Bacteriol.*, 90 (1965) 1159.
4 L. I. HOCHSTEIN AND B. P. DALTON, *J. Bacteriol.*, 95 (1968) 37.
5 R. M. BAXTER AND N. E. GIBBONS, *Can. J. Microbiol.*, 2 (1956) 599.
6 S. T. BAYLEY AND E. GRIFFITHS, *Biochemistry*, 7 (1968) 2249.
7 F. W. J. TEALE, *Biochim. Biophys. Acta*, 35 (1959) 543.
8 C. P. LEE, G. L. SOTTOCASA AND L. ERNSTER, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 33.
9 D. B. ROODYN, P. J. REIS AND T. S. WORK, *Biochem. J.*, 80 (1961) 9.
10 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
11 R. M. BAXTER, *Can. J. Microbiol.*, 6 (1960) 417.
12 D. F. WILSON, *Arch. Biochem. Biophys.*, 121 (1967) 757.
13 B. CHANCE AND E. L. SPENCER, Jr., *Discussions Faraday Soc.*, 27 (1959) 200.
14 B. CHANCE AND B. SCHOENER, *J. Biol. Chem.*, 241 (1966) 4567.
15 B. CHANCE, in J. E. FALK, R. LEMBERG AND R. K. MORTON, *Haematin Enzymes*, Part 2, Pergamon Press, New York, 1961, p. 433.
16 B. CHANCE, *Nature*, 169 (1952) 215.
17 S. S. DEEB AND P. HAGER, *J. Biol. Chem.*, 239 (1964) 1024.
18 L. VAZQUEZ-COLON AND T. E. KING, *Arch. Biochem. Biophys.*, 105 (1967) 190.

19 J. N. WILLIAMS, Jr., *Arch. Biochem. Biophys.*, 107 (1964) 537.
20 L. N. CASTOR AND B. CHANCE, *J. Biol. Chem.*, 234 (1959) 1587.
21 H. W. TABER AND M. MORRISON, *Arch. Biochem. Biophys.*, 105 (1964) 367.
22 K. S. CHEAH, *Biochim. Biophys. Acta*, 153 (1968) 718.
23 P. L. BROBERG AND L. SMITH, *Biochim. Biophys. Acta*, 131 (1967) 479.
24 C. C. YIP, *Biochim. Biophys. Acta*, 128 (1966) 262.
25 J. K. LANYI, *Arch. Biochem. Biophys.*, 128 (1968) 716.