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Electron Transport System of the Protoheme-requiring Anaerobe *Bacteroides melaninogenicus*

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Protoheme is essential for the growth of some strains of *Bacteroides melaninogenicus*. At low concentrations in the growth medium, protoheme determines the doubling time, total cell yield, and amount of cytochrome per bacterium. At high protoheme concentrations, the doubling time, total cell yield, and amount of enzymatically reducible cytochrome appear to remain nearly constant, and protoheme is accumulated by the cell. The accumulated protoheme can support the growth of the bacterium for at least eight generations in a protoheme-free medium. When growth and cytochrome content are proportional during growth at low protoheme concentrations, the bacteria incorporate 10 to 20% of the total available protoheme into a membrane-bound respiratory system. This respiratory system includes cytochrome *c*, a carbon monoxide-binding pigment, and possibly flavoproteins. The pigments can be reversibly reduced by reduced nicotinamide adenine dinucleotide or endogenous metabolism and can be oxidized anaerobically by fumarate or by shaking in air. Electron transport is inhibited by 2-*n*-nonyl-4-hydroxy-quinoline-*N*-oxide.

Many strains of *Bacteroides melaninogenicus* require heme and vitamin K for growth (4, 5, 8). The fact that both heme and vitamin K are required for growth of this obligate anaerobe suggests that an electron transport system may be involved in its metabolism. This study was initiated to determine whether a respiratory system is present in *B. melaninogenicus* and whether the level of heme supplementation in the growth medium affects the formation of the respiratory system.

The strain of *B. melaninogenicus* used in this study, strain CR_2A, required protoheme for growth. Growth of this strain in complex medium was independent of the presence of carbohydrate. Large amounts of ammonia, acetate, propionate, lactate, and butyrate are the major products of metabolism of *B. melaninogenicus* (10). In this study, we demonstrated the presence of a membrane-bound respiratory system involving flavoproteins, cytochrome *c*, and a carbon monoxide-binding pigment. These pigments were reversibly reduced by reduced nicotinamide adenine dinucleotide (NADH) and were oxidized by fumarate. The growth rate, final cell yield, and concentration of respiratory pigments were dependent on the concentration of protoheme supplied in the medium. Even though the growth of the organism was limited by the concentration of protoheme in the medium, only 10 to 20% of the added protoheme was found in the enzymatically reducible cytochrome *c*.

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MATERIALS AND METHODS

Organism. B. melaninogenicus strain CR_2A was a gift from R. J. Gibbons. Cultures were preserved by the addition of 15% (v/v) sterile glycerol during the late log phase of growth and were kept at −60 C in an atmosphere of CO_2-H_2. The criteria used to judge the purity of the cultures included phase-contrast microscopic examination of the living cells, Gram stain, and colonial morphology. The organism used in these experiments was a typical gram-negative, nonmotile cocco-bacillary form that gave small, glistening, smooth colonies which turned black in 6 to 7 days when incubated anaerobically on blood agar plates. Concomitant with pigment production, there was a zone of clearing around each colony on the blood agar plate.

Media. The growth medium consisted of 2.7% (v/v) Trypticase (BBL), 0.3% yeast extract (Difco), 34.5 mM NaCl, 14.6 mM K_2HPO_4, and 14.7 mM K_2CO_3. The medium was autoclaved for 15 min at 121 C, cooled to room temperature, equilibrated with H_2—CO_2, and stored inside Brewer jars. The stock sol-

ution of protoheme was prepared by dissolving 20 mg of protoheme in 0.5 ml of pyridine. The volume of the protoheme solution was brought to 5 ml with 0.5 M NH_4OH and was stored in the dark at −16 C. The final concentration of protoheme added to the medium is indicated in the text. The organism was streaked on blood agar which was prepared with 4.0% (w/v) Heart Infusion Agar (Difco) and 0.5% agar (BBL) that was autoclaved and cooled to 55 C prior to the addition of 10% (v/v) sterile defribrinated horse blood (Colorado Serum Co., Denver, Colo.).

Growth of the organism. Bacteria were grown at 37 C in flasks or screw-cap centrifuge tubes inside Brewer jars in an atmosphere of 5% CO_2 and 95% H_2 (v/v). A red-hot nichrome wire was used to catalyze water formation from residual oxygen in the Brewer jars. The wire was kept hot for at least 30 min prior to incubation. All media were stored in these jars and were equilibrated with the CO_2-H_2 atmosphere before use. Unless a sufficiently large inoculum was used, it was difficult to obtain reproducible bacterial growth. In these experiments, the inoculum used was 10% of the final volume of the culture. The inoculum was prepared by transferring the cells to medium without added protoheme and allowing the cells to grow to a maximal absorbance. This was repeated until no further growth was observed. Under these conditions, the inoculum used in the third transfer would not grow unless protoheme was present in the medium.

Preparation of cell suspensions. Cultures were harvested in the late log phase of growth by centrifugation at 14,000 × *g* for 30 min. The bacterial pellet was washed and suspended in 50 mM phosphate buffer (*p*H 7.6) that contained 1 mM dithiothreitol and 10.4 mM sodium thioglycolate. The buffer was deoxygenated with nitrogen or CO_2.

Determination of the doubling time. The doubling time was defined as the time necessary for the absorbance to double between bacterial densities of 0.07 and 0.35 mg (dry weight)/ml. The absorbance was measured in round tubes, 13 mm in diameter, with a Spectronic-20 colorimeter (Bausch & Lomb, Rochester, N.Y.) at 750 nm.

Protein. Protein was determined colorimetrically (9) with bovine serum albumin as the standard.

Difference spectra. Samples from the same bacterial suspension having respiratory pigments at two different oxidation levels were compared by difference spectroscopy. Difference spectra were measured with a Cary 14 CM spectrophotometer as described previously (14, 16). To obtain reduced minus oxidized difference spectra, the respiratory pigments in one part of a bacterial suspension were allowed to reduce by endogenous metabolism. In another portion of the same bacterial suspension, the respiratory pigments were oxidized before measurement by vigorously shaking in air or by the anaerobic addition of fumarate. The measurement of reduced minus oxidized difference spectra was established as an accurate measure of cytochrome content by comparing difference spectra to the absolute spectrum of bacteria. The absolute spectrum was obtained by measuring the bacteria with endogenously reduced pigments against ground glass of appropriate light scattering properties (14). The carbon monoxide-reduced minus reduced difference spectra were measured in the following way. The respiratory pigments in a bacterial suspension were allowed to reduce by endogenous metabolism. A portion of the suspension was saturated with carbon monoxide and was compared to a second portion of this suspension.

Cytochrome *c* was measured as the absorbance increment between the α maximum at 555 nm and the point at 555 nm on a base line connecting points at 600 and 537 nm in the reduced minus oxidized difference spectrum (unless otherwise stated). Substrates such as NADH, succinate, or formate were added to anaerobic suspensions in Thunberg cuvettes or under scrubbed nitrogen as described (16). Spectra at the temperature of liquid nitrogen were measured essentially as described by Estabrook (1), in an apparatus employing Lucite cuvettes with a 2-mm path length. This apparatus was designed by T. Orr.

Pyridine hemochrome. Pyridine hemochromes were measured as described by Falk (2).

Extraction of hemes. To extract protoheme from the bacteria, cold acetone, water and concentrated HCl (50:10:2.5, v/v) were added to the bacterial pellet, and the suspension was homogenized with a Teflon homogenizer and then centrifuged in polypropylene tubes. Approximately 40 ml of acid acetone was used per *g* (dry weight). The residue was suspended in water, and the heme *c* was determined by preparing the pyridine hemochrome. The supernatant portion of the acid acetone extraction was then extracted with ether, and the ether was washed with 0.27 M HCl. The aqueous layer was then extracted again with more ether. The porphyrin was separated from the protoheme by extracting the combined ether phases with 4.5 M HCl. The porphyrin was then taken up by another portion of ether by adjusting the HCl to *p*H 5 with $NaHCO_3$, and the absorbance was measured after extraction with 1.5 M HCl. The porphyrin spectrum had absorption maxima at 601, 556, and 409 nm, with a ratio of the maxima at 409 to 556 nm of 19.4 [the ratio expected for protoporphyrin is 19.3 (2)]. The concentration of protoporphyrin was calculated with $\epsilon_{409} = 262 \times 10^3$ per mole per cm (2). The ether layer containing protoheme was washed with two portions of 0.27 M HCl, and the ether was evaporated to dryness in vacuo. The reduced pryidine hemochrome was then prepared.

Materials. Materials were the best grade commercially available, as reported previously (15).

RESULTS

Protoheme requirement for growth. The complex medium necessary for growth of *B. melaninogenicus* contained less than 3.7 pmoles of protoheme/ml, as measured with the medium at 10 times the usual concentration. In this medium, *B. melaninogenicus* was unable to grow unless protoheme was added, provided that the bacteria were depleted of heme by incubation in the absence of heme until growth stopped. Bacteria that were depleted of heme showed an increase in both the

final cell yield and in the doubling time when the protoheme supplementation of the medium was increased between <3.7 and 615 pmoles/ml. At protoheme concentrations above 615 pmoles/ml, the total cell yield remained near 0.9 mg (dry weight)/ml and the doubling time between 3 and 4 hr (Fig 1).

Cytochrome system. The respiratory pigments of a suspension of *B. melaninogenicus* can be oxidized by shaking the bacteria in air or by the anaerobic addition of fumarate in a Thunberg cuvette. The reduced minus oxidized difference spectrum is shown in Fig 2. The spectrum was the same whether oxidation was achieved by shaking in air or by the anaerobic addition of fumarate. In the suspension in which the respiratory pigments were oxidized by shaking in air, the pigments were reduced in 20 min by the endogenous metabolism of the bacteria at room temperature. A cytochrome with maxima at 555, 528, and 422 nm could be detected in the reduced minus oxidized difference spectrum. A minimum at 450 nm in this spectrum suggested the involvement of flavoproteins and possibly of nonheme iron pigments in this respiratory system. A carbon monoxide-binding pigment, with maxima at 578, 540, and 416 nm and with minima at 553 and 425 nm, could be detected in the carbon monoxide minus reduced difference spectra. The respiratory pigments were reduced by the endogenous metabolism of the bacteria. In bacteria grown in the presence of less than 0.60 nmole of protoheme/ml, in which the respiratory pigments were reduced by endogenous metabolism, there was no further reduction of the cytochrome system when $Na_2S_2O_4$ was added.

Localization of the cytochrome system. B. melaninogenicus could be ruptured, as determined by phase-contrast microscopy, by exposure to ultrasonic vibration for 4 min. This exposure was performed under nitrogen and the temperature was kept below 10 C. The cell-free preparation was then centrifuged at 25,000 × *g* for 30 min, the supernatant portion was decanted, and the pellet was resuspended in the phosphate-thioglycolate buffer. The resuspended particulate preparation contained 99% of the cytochromes present in the intact bacteria. Less than 0.1% of the cytochromes could be detected in the supernatant fluid.

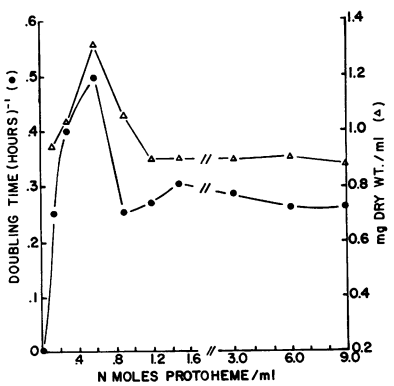

FIG. 1. *Doubling time and final yield of Bacteroides melaninogenicus with different levels of protoheme. The inocula consisted of cells depleted of protoheme. Dry weight and doubling time were determined as described in Materials and Methods.*

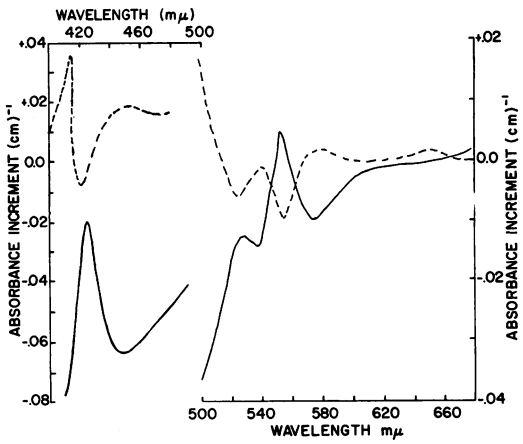

FIG. 2. *Difference spectra of Bacteroides melaninogenicus. Bacteria were suspended at a density of 6.1 mg of bacterial protein/ml in 50 mM phosphate buffer (pH 7.6) containing 1 mM dithiothreitol, 10.4 mM sodium thioglycolate, and 15% glycerol. The buffer was deoxygenated with nitrogen. Symbols: Solid line, bacteria with pigments reduced by endogenous metabolism compared to bacterial pigments oxidized by vigorous shaking in air; dashed line, bacteria with pigments reduced by endogenous metabolism and saturated with carbon monoxide compared to bacteria with pigments reduced by endogenous metabolism.*

Respiratory system. The respiratory pigments of the resuspended particulate preparation could be completely reduced in the presence of 5 mM NADH. The pigments were not reduced in the presence of succinate, formate, DL-lactate, or hydrogen. In the presence of 1 μM NADH, the anaerobic addition of 20 mM fumarate resulted in oxidation of the pigments. The reduction of the respiratory pigments in the presence of NADH was completely inhibited in the presence of 2 mM 2-*n*-nonyl-4 hydroxyquinoline *N*-oxide. The reduced minus oxidized difference spectrum of the membrane preparation at room temperature and at −190 C is illustrated in Fig. 3. At −190 C, the maxima of the cytochrome were at 550, 520,

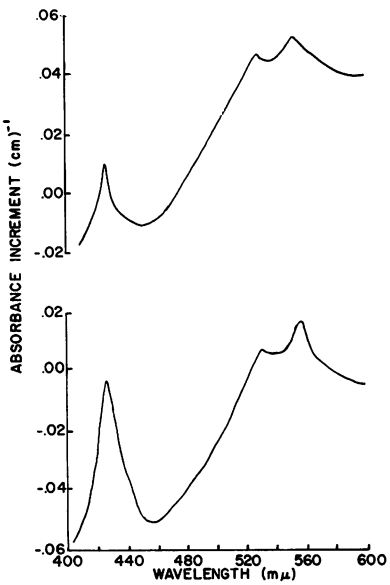

FIG. 3. *Difference spectrum of a cell-free membrane preparation of Bacteriodes melaninogenicus. Pigments in membrane preparations reduced in the presence of 5 mM NADH were compared to pigments in membrane preparations oxidized in the presence of 20 mM fumarate. The membranes were suspended in the buffer described in Fig. 2 at a concentration of 10.3 mg (dry weight)/ml. Upper curve indicates the difference spectrum measured at −190 C in Lucite cuvettes of 2-mm path length. Lower curve indicates a difference spectrum of a part of the same preparation measured at 25 C in cuvettes of 10-mm path length.*

and 428 nm. There was no evidence of splitting of the α maximum.

Identification of the hemes of the respiratory system. B. melaninogenicus was grown to a density of 0.45 mg (dry weight)/ml in the presence of 77 pmoles of protoheme/ml. The low yield of cells indicates that growth was limited by this protoheme concentration (Fig. 1). In another experiment, the bacteria were grown to a density of 0.9 mg (dry weight)/ml in 1,550 pmoles of protoheme/ml. In this case, the protoheme concentration did not limit growth. The bacteria were harvested and extracted with acid acetone. The protoheme and protoporphyrin in the acid acetone extract and the heme *c* in the residue from the extraction were measured (Table 1). The heme *c* in the residue, measured as the reduced pyridine hemochrome, was equivalent to the heme *c* calculated from the difference spectrum of whole cells. In both experiments, more heme was found in the reduced pyridine hemochrome of the intact bacteria than was enzymatically reducible as cytochrome *c*. This indicates that there is protoheme in the bacteria that is not enzymatically reducible.

TABLE 1. *Hemes in Bacteroides melaninogenicus grown in the presence of protoheme*

Heme	Amt of protoheme bacteria grown with	
	77 pmoles/ml	1,550 pmoles/ml
Enzymatically reducible cytochrome *c*[a]	23.6[b]	80.0
Total heme in bacteria	30.6	143.0
Protoheme extracted	0.05	89.0
Protoporphyrin extracted	31.3	73.2
Heme *c* in residue after extraction	22.3	78.0

[a] Enzymatically reducible cytochrome *c* was determined from the reduced minus oxidized difference spectra, as in Fig. 2, and was calculated with $\epsilon_{553} = 20.0 \times 10^3$ per mole per cm. The measurement of hemes and porphyrins is described in Methods and Materials. Total heme in the bacteria represents the heme measured as the reduced pyridine hemochrome of the intact bacteria. This measurement includes both heme *c* and protoheme.

[b] Amount of heme, expressed as nanomoles per gram (dry weight).

Effect of protoheme concentration on cytochrome formation. The effect of different levels of protoheme in the medium on the concentration of enzymatically reducible cytochrome *c* is shown in Fig 4. The cytochromes were determined after the bacteria had reached their maximal cell density. The concentration of enzymatically reducible cytochrome *c* and of the carbon monoxide-binding pigment increased as the protoheme concentration increased to approximately 0.5 μg of protoheme/ml. At this concentration, a maximal level of enzymatically reducible cytochrome was found to be reproducible, although the absolute concentration of pigments varied somewhat. At higher protoheme concentrations, the level of enzymatically reducible cytochrome decreased slightly. The enzymatically reducible cytochrome formed with various levels of protoheme supplementation paralleled the changes in doubling times and final cell yields, as illustrated in Fig 1.

Growth with excess protoheme. When *B. melaninogenicus* was grown in the presence of 1.5 nmoles of protoheme/ml for several generations and then was transferred to medium that did not contain protoheme, the bacteria were capable of at least eight divisions before growth stopped. When protoheme was added to this culture, growth began immediately.

As the protoheme content of the medium was

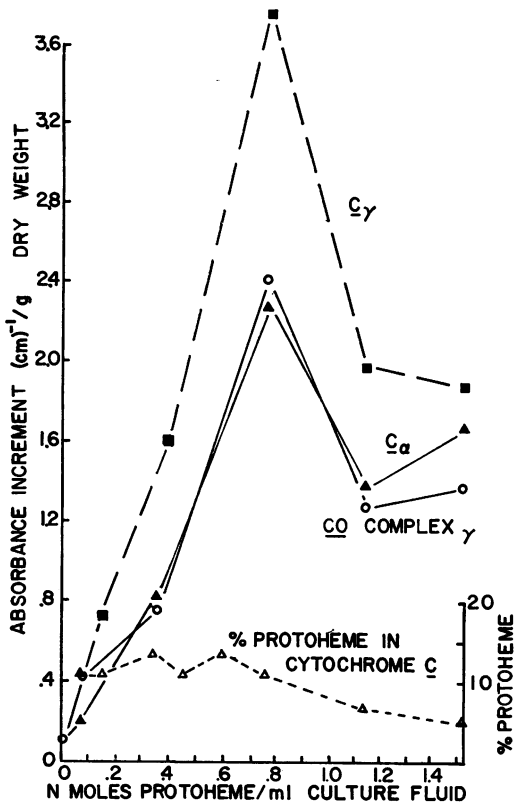

FIG. 4. *Cytochrome content of Bacteroides melaninogenicus grown with increasing concentrations of protoheme in the medium. The cytochromes were measured from the reduced minus oxidized difference spectra as in Fig. 2. Symbols: ▲, the absorbance increment between the α maxima at 555 nm and the minimum at 539 nm; ■, the absorbance increment between the γ maximum at 416 nm and the trough at 450 nm in the cells with pigments reduced by endogenous metabolism versus cells with pigments oxidized in air; ○, the absorbance increment between the γ maximum at 416 nm and the trough at 425 nm in the difference spectrum of a suspension with pigments reduced by metabolism and saturated with carbon monoxide compared to a suspension with pigments metabolically reduced. All results are plotted per milligram, dry weight. In the lower portion of the figure, the percentage of the protoheme in the medium at the beginning of the experiment that was subsequently found as enzymatically reducible cytochrome c is shown (△). The right hand axis refers to this percentage.*

increased, the cells examined after centrifugation become darker in color. Cells grown on blood agar plates (containing approximately 0.2 μmole of protoheme/ml) formed the characteristic shiny black colonies which give the bacteria its name. The black color has been known to be due to protoheme for a long time (11). Black colonies formed during an 8-day incubation period were scraped from the blood agar plates and were washed twice with 50 mM phosphate buffer (*p*H 7.). The concentration of protoheme was determined by preparing the reduced pyridine hemochrome of a suspension of the washed pellet in the buffer. About 43% of the dry weight of the bacteria was protoheme. The viability of the cells in the colonies decreased as the cell mass became darker.

Efficiency of protoheme utilization. When the growth rate, final cell yield, and cytochrome content were proportional to the protoheme concentration in the growth medium, between 10 and 20% of the available protoheme was found as enzymatically reducible cytochrome *c* (lower part of Fig 4). Less than 0.01% of the initial protoheme could be detected in the medium after a bacterial growth cycle. At protoheme concentrations greater than 761 pmoles/ml, a smaller proportion of the total protoheme in the growth medium was found as enzymatically reducible cytochrome *c* and a progressively larger proportion was found as nonenzymatically reducible protoheme.

During growth, *B. melaninogenicus* produces an agent that can destroy protoheme. This activity can be measured by adding protoheme to the spent medium after the cells have been removed and monitoring the decrease in protoheme with time; the protoheme is determined as the reduced pyridine hemochrome. The protoheme-destroying activity can be eliminated by boiling the medium, lowering the *p*H to 2.0, or vigorously aerating the medium before adding the protoheme. In our experiment the protoheme-destroying activity decreased proportionately with dilution of the medium. In an experiment in which 90% of the added protoheme had disappeared, we were able to recover approximately 25% of the protoheme when the medium was vigorously aerated before the pyridine hemochrome was prepared. This suggested that part of the protoheme is reduced to a nondetectable compound, which becomes detectable after oxidation in air. Measured as ultraviolet fluorescence after extraction with ethyl acetate and acetic acid (3:1, v/v) (2), no prophyrins could be detected in the medium after the heme had disappeared.

DISCUSSION

The metabolism of the obligate anaerobe *B. melaninogenicus* appears to require the presence of a functional membrane-bound electron transport system. The formation of this electron transport system is dependent on the availability of protoheme in the growth medium. Once the or-

ganism has depleted its internal stores of heme, growth is dependent on added protoheme. The growth rate, total cell yield, and the level of both cytochrome *c* and the carbon monoxide-binding pigment are dependent on the protoheme concentration supplied in the medium. In three heme-requiring species of *Haemophilus* (13), the total cell yield, cytochrome *b*, cytochrome oxidase *o*, and NADH oxidase flavoprotein are dependent on the protoheme concentration supplied in the growth medium.

The function of the electron transport system in the metabolism of *B. melaninogenicus* remains obscure. Both NADH, a reductant, and fumarate, an electron acceptor, have thus far been shown to react with the particulate electron transport system. Oxygen acts as an electron acceptor but is lethal to these organisms. Another heme-requiring anaerobe, *B. ruminicola*, contains a membrane-bound electron transport system that is reduced in the presence of NADH and oxidized in the presence of fumarate (17). The metabolic processes in *B. melaninogenicus* by which NADH is generated may result from the metabolism of amino acids. In preliminary experiments, the addition of carbohydrates to the growth medium did not result in faster growth rates, and very little of the carbohydrate was utilized during growth. The organism produced large amounts of ammonia and 2, 3, and 4 carbon acids during growth. A very active collagenase is also released during the growth cycle (6). These findings are consistent with the hypothesis that amino acids are important in the metabolism of this organism.

The enzymatically reducible cytochrome *c* can be quantitatively accounted for as the heme *c* that remains in the residue after acid acetone extraction of the bacteria. When the bacteria are grown in protoheme concentrations that limit growth, protoporphyrin is found in the bacteria. Some of this protoporphyrin may represent protoheme that was deironed in the acid acetone extraction process. The conditions of extraction of the hemes, which are acid and highly reducing, favor deironing of the hemes (2). Some protoheme exists in the cells, even when they are grown at low protoheme concentrations, as the total heme measured as the reduced pyridine hemochrome exceeds the heme *c*. The protoheme could represent the prosthetic group of the carbon monoxide-binding pigment, which has the spectral properties of cytochrome oxidase *o* (12). This pigment is thought to contain protoheme as its prosthetic group. Since the α maxima of cytochrome *o* and cytochrome *c* in the reduced minus oxidized difference spectra almost completely overlap and the heme *c* is equivalent to the enzymatically reducible cytochrome *c*, it is unlikely that there is much cytochrome oxidase *o* in this organism. Since the concentration of the carbon monoxide-binding pigment closely parallels that of the cytochrome *c* (Fig. 4), there is a strong possibility that the cytochrome *c* reacts with carbon monoxide. Other bacterial cytochrome *c* pigments have been reported to react with carbon monoxide (7, 18). There is no evidence for enzymatically reducible cytochrome *b* in this strain of *B. melaninogenicus*. When the bacteria were grown at high protoheme concentrations, protoheme could be recovered from the bacteria. This protoheme was not enzymatically reducible and did not combine with carbon monoxide unless it was chemically reduced. Strain CR_2A of *B. melaninogenicus* differs somewhat from one strain described earlier by C. A. Reddy and M. P. Bryant (Bacteriol. Proc., p. 40, 1967). These investigators found that one strain of *B. melaninogenicus* contained cytochrome *c* and a second strain contained cytochromes *b* and *o*.

During growth, *B. melaninogenicus* generates a substance that destroys protoheme that is added to the spent medium. This substance has the properties of a powerful reducing agent. Part of the protoheme that is lost can be recovered by aerating the medium, and the products of the protoheme-destroying agent are apparently not porphyrins. The protoheme-destroying agent probably accounts for the fact that only 10 to 20% of the protoheme available in the medium at the beginning of the growth cycle can be recovered in the cytochrome *c* when the growth of the bacteria is limited by the concentration of protoheme added to the medium.

The highest concentration of cytochrome *c* found in *B. melaninogenicus*, 0.11 nmole/mg (dry weight), corresponds to three times the concentration of cytochrome *b* plus cytochrome *o* found in the heme-requiring anaerobe *B. ruminicola* (17) and is equal to the level of these cytochromes found in aerobically growing *Staphylococcus aureus* (3). The level of cytochrome *c* in *B. melaninogenicus* is 10% of that found in anaerobically growing *H. parainfluenzae*.

We have previously reported (P. R. Courant and D. C. White, Bacteriol. Proc., p. 105, 1967) that strain BE-1 is capable of growth in the absence of added protoheme. The bacteria used in this study contained a slightly larger gram-negative cocco-bacillary organism which apparently supplied minimal protoheme to strain BE-1. This organism had a very long doubling time and apparently limited the growth of strain BE-1 to this very slow rate. All attempts to isolate a pure culture of strain BE-1 failed, so the strain was discarded. The present study was performed with strain CR_2A. Strain CR_2A gave no evidence

of variation and was absolutely dependent on protoheme in the medium, as documented in this paper.

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