JOURNAL OF BACTERIOLOGY, Sept. 1970, p. 714-721

Vol. 103, No. 3
Printed in U.S.A.

Cytochrome Content of Two Pseudomonads Containing Mixed-Function Oxidase Systems

JULIAN A. PETERSON

Department of Biochemistry, The University of Texas (Southwestern) Medical School at Dallas, Dallas, Texas 75235

Received for publication 16 June 1970

The cytochrome and nonheme iron protein content of two pseudomonads, *Pseudomonas oleovorans* and *P. putida*, containing mixed function oxidase systems was examined. The mixed function oxidase system of *P. oleovorans* and *P. putida* had previously been shown to be present in cells which had been grown on hexane and camphor, respectively, as energy source. The content of protoheme was found to increase significantly when the organisms were grown on the substrates for mixed function oxidation. The nonheme iron protein content increased significantly in the case of *P. putida* and was constant in *P. oleovorans*. The cytochrome *c* content was essentially constant in both pseudomonads. The content of cytochrome P-450 in *P. putida* increased from an immeasurably low amount to 0.15 nmoles per mg (dry weight). The content of cytochrome *o* in *P. oleovorans* increased by a factor of 4.5. *P. oleovorans* was not found to contain detectable quantities of cytochrome P-450 either in the presence or absence of the mixed function oxidase system.

Cytochrome content has been found to vary markedly with growth conditions in numerous bacteria (6, 22). However, the nature and function of these various cytochromes were extensively studied for only a few types of bacteria (6, 22). For example, the respiratory pigments of *Micrococcus denitrificans* (21) and *Bacillus subtilis* (3) were shown to be similar in many respects to the respiratory pigments of mammalian mitochondria, in that these bacteria contain cytochromes of the *a*, a_3, *c*, and *b* types. In addition, their sites of phosphorylation also appear to be similar to those observed with mammalian mitochondria (11).

Cytochrome *o*, which is characterized by its ability in the reduced form to combine with carbon monoxide, has been found to be present in many different strains of bacteria under a variety of growth conditions (3). Photochemical dissociation of the carbon monoxide complex of the reduced form of cytochrome *o* has shown that it can function as a terminal oxidase in some bacteria (3). The carbon monoxide difference spectrum of the reduced form of cytochrome *o* has an absorption maximum at 419 nm and a minimum at 436 nm (3). The functional group of cytochrome *o* has been previously characterized as protoporphyrin IX (23). The present study was undertaken to determine the possible function of cytochrome *o* as a terminal oxidase and the relationship of this cytochrome with other bacterial respiratory pathways.

The pseudomonads selected for this study were chosen because they were previously shown to contain mixed function oxidase systems (12, 16, 24). *Pseudomonas oleovorans* contains an inducible mixed function oxidase system which is specific for the ω-oxidation of fatty acids and hydrocarbons (13). The ω-hydroxylase system was shown to involve a reduced nicotinamide adenine dinucleotide (NADH)-dependent rubredoxin reductase, rubredoxin (a nonheme iron protein not containing labile sulfur), and the ω-hydroxylase (16). The nature of the functional group of the ω-hydroxylase is not known; however, previous studies indicated that cytochrome P-450 is not involved (19).

The metabolism of camphor by *P. putida* was shown to involve several mixed function oxidase steps (12, 24). The camphor methylene hydroxylase system was found to require a NADH-dependent putidaredoxin reductase (a flavoprotein), putidaredoxin (a nonheme iron protein containing labile sulfur), and the methylene hydroxylase (12). The functional group of the methylene hydroxylase was shown to be a type of cytochrome P-450 (12). The cytochrome P-450-containing camphor methylene hydroxylase system is similar in many ways to the adrenal mitochondrial steroid hydroxylase system (15), with the major exception

that the cytochrome P-450 of *P. putida* is truly soluble and quite stable in water solution at 4 C (12), whereas the adrenal mitochondrial cytochrome P-450 is membrane-bound and is solubilized only with great difficulty (4).

This paper will correlate cytochrome and nonheme iron protein concentration changes in these two pseudomonads with the induction of their mixed function oxidase systems.

MATERIALS AND METHODS

Growth of organisms. *P. oleovorans* was grown on a medium containing the following components per liter: $(NH_4)_2SO_4$, 7.6 mmoles; KH_2PO_4, 3.7 mmoles; Na_2HPO_4, 24.6 mmoles; $MgSO_4$, 0.81 mmoles; $CaCl_2$, 0.18 mmoles; $FeCl_3$, 0.07 mmoles; and supplemented with 0.02 g of yeast extract per liter. The final *p*H of the growth medium was 7.10. A sterile, concentrated solution of glucose was added to the medium to give a final concentration of 17 mM (3.0 g/liter). Technical-grade hexane (15 ml, >95% pure; Phillips Petroleum Co.) was layered on 500 ml of the growth medium when it was used as carbon source. *P. putida* was grown on a medium containing the following concentrations of each component per liter: NH_4Cl, 37 mmoles; K_2HPO_4, 47 mmoles; KH_2PO_4, 22.8 mmoles; $MgSO_4$, 0.81 mmoles; $CaCl_2$, 0.18 mmoles; $FeCl_3$, 0.07 mmoles; and supplemented with 0.1 g of yeast extract and tryptone per liter. The final *p*H of the growth medium was 7.0. Sterile glucose was added as described above for those cells grown on glucose. Solid camphor [2.0 g, research grade d(+) camphor; J. T. Baker Co.] was crushed and it floated on the surface of 500 ml of the growth medium when used as carbon source.

Both organisms were grown in 2-liter Erlenmeyer flasks on a rotating-platform shaker at 30 C. The flasks containing 500 ml of growth medium were inoculated with cells which were grown overnight to the stationary phase on 17 mM glucose. The flasks were shaken until the cells reached late-log-phase growth as determined turbidimetrically at 600 nm. The cells were harvested by centrifugation at 8,000 × *g* for 5 min and washed three times with 50 mM potassium phosphate buffer (*p*H 7.4). The cells were finally suspended in the phosphate buffer and used immediately for the metabolic determinations. The cells were stored and frozen at −20 C if they were to be used for cytochrome or spectral analysis. Storage at −20 C does not result in any measurable change in the cytochrome composition.

The yield of the cells obtained was determined by a dry-weight analysis. The whole cells were precipitated with 70% ethanol and the extract was discarded. The precipitate was dried at 120 C to a constant weight. All weights of cells are expressed as milligrams of dry weight.

Cytochrome analysis. The cytochrome extraction procedure is similar to the procedure of Rieske (20). In all cases, at least 100 mg of cells containing at least 30 nmoles of each cytochrome was used for the extraction. All steps, except as noted, were performed in the dark at 0 C as rapidly as possible to prevent decomposition of the extracted protoheme. All of the centrifugations were done with a desk-top clinical centrifuge at 3,000 rev/min for 5 min at room temperature.

The whole cells were treated with three volumes of acetone and centrifuged, and the extract was discarded. The precipitate was suspended in 5 ml of chloroform:methanol (2:1) by using a homogenizer and centrifuged, and the extract was discarded. The precipitate was suspended in 5 ml of acetone and centrifuged, and the extract was discarded. The precipitate was extracted twice with 3 ml of 1% HCl in acetone (freshly prepared) and centrifuged. The pooled extracts were concentrated almost to dryness by using a rotatory evaporator at 20 C. Immediately after concentration, 6 ml of a solution containing 0.1 N KOH and 20% pyridine was added to each sample, and the reduced minus oxidized difference spectrum was determined. The difference spectra were recorded at room temperature with an Aminco-Chance dual-wavelength, split-beam spectrophotometer in the split-beam mode. The acid acetone extract contains the ferric form of protoporphyrin IX. An ξ_M value of 34.1×10^3 was used for the reduced minus oxidized difference spectrum at 557 nm minus 575 nm of the pyridine hemochromogen derivative (20). If there were any heme *a* cytochrome present in these bacteria, the heme would have been found in the acid acetone extract; however, not a trace of heme *a* was detected at 587 nm in the pyridine homochromogen derivatives of the acid acetone extracts. The residue from the acid acetone extract was suspended in 6 ml of the alkaline pyridine mixture, and the reduced minus oxidized difference spectrum was determined. The content of *c*-type cytochrome present in the residue was calculated using an ξ_M value of 21.0×10^3 for the reduced minus oxidized difference spectrum at 550 nm minus 540 nm (20). The extraction procedure was shown to give a recovery of greater than 90% of *b*- and *c*-type cytochromes present in electron transport particles prepared from beef heart mitochondria, and the recovery of cytochromes is presumed to be similar from the bacteria.

Cytochrome *o* was determined in the whole cells by measuring the change in absorption at 419 nm relative to 490 nm when carbon monoxide was bubbled through the suspension of $Na_2S_2O_4$ treated cells. An ξ_M value of 80×10^3 was used for the calculation of the content of cytochrome *o* (3). Cytochrome P-450 was determined in whole cells by measuring the change in absorption at 446 nm relative to 490 nm when carbon monoxide was bubbled through a suspension of $Na_2S_2O_4$-treated cells. An ξ_M value of 210×10^3 was used for the calculation of the content of cytochrome P-450 (10).

EPR spectra. The electron paramagnetic resonance (EPR) spectra were recorded as described in the figure legends by using a Varian E4 EPR spectrometer equipped with a variable-temperature attachment. The concentration of bacterial cells was adjusted to 30 mg/ml in each case. The concentration of nonheme iron protein was determined with standard EPR tubes which were calibrated with known concentrations

of adrenodoxin. The area of the original EPR absorption spectra was obtained by using a computer program developed by L. Peterson. The areas of these integrated EPR spectra were plotted versus the amount of adrenodoxin in the standard samples; the response of the EPR spectrometer was found to be linear over a 200-fold concentration range. The results obtained were reproducible to better than ±2% by using similar samples of either bacterial cells or adrenodoxin.

Metabolic studies. The respiratory capability of these bacteria was determined with a Clark-type oxygen electrode at room temperature. The cells were prepared and washed as described in the section on growth of bacteria. If the level of endogenous respiration was too high, the cells were resuspended in 50 mM phosphate buffer (*p*H 7.4) to 25% of their original volume. The cells were aerated for 15 min at room temperature on the rotating shaker, centrifuged, and washed once before use. Each of the water-soluble substrates was added to the reaction mixture after the rate of endogenous respiration had been determined. The final concentration of the substrates was 10 mM. The *p*H of the substrates was adjusted to about 7.0 before use.

RESULTS

Metabolic studies. The effect of glucose, camphor, or hexane as the carbon source for growth on the ability of the pseudomonads to respire on various compounds is shown in Table 1. Growth of *P. oleovorans* on hexane results in the ability of this organism to respire on hexane. The large increase in the ability to respire on ethanol may be due to the presence of an alcohol dehydrogenase similar to the one induced in *P. aeruginosa* when grown on heptane (1). *P. oleovorans* grown on glucose or hexane did not possess the ability to respire on camphor. However, this organism would not also grow on camphor as the sole carbon source.

P. putida grown on camphor has the ability to respire on camphor, whereas the cells grown on glucose do not possess the ability to respire on camphor (Table 1). The sample of cells used for the respiratory experiment contained 0.15 nmoles of cytochrome P-450 per mg (dry weight) of cells. The maximal turnover for cytochrome P-450 under these conditions in whole bacterial cells must be at least 220 μmoles of O_2 consumed per min per μmole of cytochrome P-450.

Spectral analysis. The room-temperature difference spectra for glucose- and hexane-grown *P. oleovorans* are shown in Fig. 1 and 2, respectively. The difference spectra shown in Fig. 1 and 2 were recorded at identical concentrations. The noticeable difference in the visible region in the reduced minus oxidized difference spectra is because the cells grown on hexane appear to have a slightly larger content of cytochromes. This difference between the two types of cells becomes more apparent in the Soret region of the spectrum. The Soret band of the reduced minus oxidized difference spectrum is significantly larger in the cells grown on hexane. The $Na_2S_2O_4$-reduced plus carbon monoxide minus $Na_2S_2O_4$-reduced difference spectrum shows a major change in the content of cytochrome *o* in cells grown on hexane. The cytochrome *o* in cells grown on glucose is just barely detectable, whereas in cells grown on hexane the content of cytochrome *o* is 0.18 nmoles per mg (dry weight).

Table 2 is a compilation of the cytochrome content of *P. oleovorans* grown on glucose or hexane. There is not a large change in the content of cytochrome *c* under these conditions, probably as a result of the fact that the *c*-type cytochrome is a component of the constitutive membrane-bound respiratory system. An increase in the content of protoheme in these cells is found when the cells are grown on hexane as carbon source. There is a large increase in the content of cyto-

TABLE 1. *Respiratory activities*[a]

Substrate	*P. oleovorans*		*P. putida*	
	Glucose-grown	Hexane-grown	Glucose-grown	Camphor-grown
Glucose	9.3	9.0	23.0	7.7
Ethanol	9.3	38.5	20.0	10.3
L-Malate	31.0	32.4	42.4	19.3
Citrate	0	6.7	4.5	4.6
α-Ketoglutarate	5.2	14.4	13.4	7.7
Succinate	7.8	24.1	13.4	21.6
Acetate	0	7.7	4.5	0
Hexane	0	15.0		
Camphor			0	33.4

[a] Rates expressed are the difference between the rate of oxygen uptake in the presence of the substrate minus the rate in the absence of the substrate. Rates of endogenous respiration were as follows: *Pseudomonas oleovorans*, glucose grown, 19.0 nmoles of O_2 per min per mg; *P. oleovorans*, hexane grown, 14.0 nmoles of O_2 per min per mg; *P. putida*, glucose grown, 15.0 nmoles of O_2 per min per mg; *P. putida*, camphor grown, 15.0 nmoles of O_2 per min per mg. The whole cells were diluted to a final volume of 3.2 ml with 50 mM potassium phosphate buffer (*p*H 7.4). Approximately 3 to 4 mg (dry weight) of cells was used for each experiment. The substrates were added to the reaction mixture to give a final concentration of 10 mM except in the case of hexane which was added as 30 μliters of a suspension dispersed with ultrasonic oscillation and camphor which was added as camphor-saturated buffer (camphor concentration 8 mM). Values expressed as nanomoles of oxygen per minute per milligram.

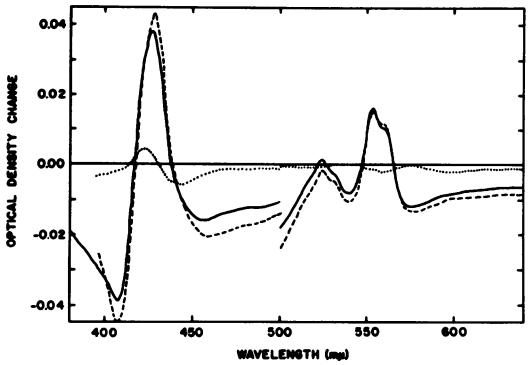

FIG. 1. *Difference spectra of glucose-grown P. oleovorans. The whole cells were diluted with 0.1 M potassium phosphate buffer (pH 7.4). The final concentration used for the visible region of the spectrum was 5 mg (dry weight) of cells per ml. The Soret region was recorded by using 2 mg (dry weight) of cells per ml. The substrate-reduced minus oxidized difference spectrum (solid line) was recorded by using 10 mM malate as the substrate. A few milligrams of solid $Na_2S_2O_4$ was added to the cuvette containing malate and the bacterial cells for the Na_2S_2O -reduced minus oxidized difference spectrum (dashed line). After the $Na_2S_2O_4$-reduced minus oxidized spectrum was recorded, the contents of the two cuvettes were mixed and the base line recorded. Carbon monoxide was bubbled through the sample cuvette for 15 sec and the $Na_2S_2O_4$-reduced plus carbon monoxide minus reduced difference spectrum (dotted line) was recorded.*

chrome *o* in hexane-grown cells. The content of nonheme iron protein in both types of cells is found to be very low and does not change when the cells are grown on glucose or hexane. The constitutive respiratory system does not contain a heme *a*-type cytochrome as the terminal oxidase when the organism is grown on either carbon source.

The room-temperature difference spectra of glucose- and camphor-grown *P. putida* are shown in Fig. 3 and 4, respectively. A large difference in the cytochrome content of *P. putida* cells grown under these conditions is immediately apparent. The changes in the Soret region of the substrate or $Na_2S_2O_4$-reduced minus oxidized difference spectra cannot readily be correlated with actual changes in the cytochrome content, probably because cytochrome P-450 has a very anomalous absorption in this region (10). The $Na_2S_2O_4$-reduced plus carbon monoxide minus $Na_2S_2O_4$-reduced difference spectra of glucose-grown cells indicates that they contain cytochrome *o*. Cytochromes of the heme *a* type were found to be totally absent from *P. putida* when grown on either glucose or camphor. The camphor-grown cells contain a carbon monoxide-binding pigment other than cytochrome *o*, as evidenced by the large increase in absorption at 446 nm in the $Na_2S_2O_4$-reduced plus carbon monoxide minus $Na_2S_2O_4$-reduced difference spectrum. This cytochrome has been shown to be required for camphor methylene hydroxylation and has been called cytochrome $P\text{-}450_{CAM}$ (12).

The changes in *P. putida* which are observed in the visible region of the spectrum can be correlated with actual changes in cytochrome content determined chemically (Table 3). The content of cytochrome *c* was found not to change greatly in those cells grown on camphor. The content of cytochrome *c* in *P. putida* was also determined by using the reduced minus oxidized difference spectrum of whole camphor-grown cells. The

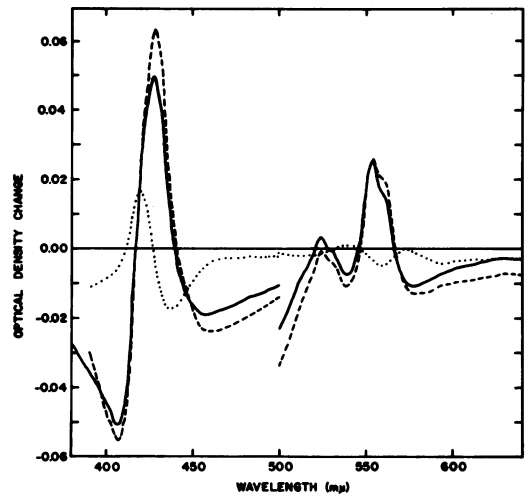

FIG. 2. *Difference spectra of hexane-grown P. oleovorans. The substrate reduced minus oxidized (solid line), $Na_2S_2O_4$-reduced minus oxidized (dashed line), and $Na_2S_2O_4$-reduced plus carbon monoxide minus reduced (dotted line) difference spectra were recorded as described in the legend to Fig. 1.*

TABLE 2. *Cytochrome content of Pseudomonas oleovorans*[a]

Cytochrome	Glucose-grown	Hexane-grown
Cytochrome *c*	0.27	0.25
Protoheme	0.14	0.21
Cytochrome *o*	0.04	0.18

[a] The content of cytochrome *c* and protoheme was determined essentially as described by Rieske (2) and as outlined in the text. The content of cytochrome *o* was determined from the absorption change at 419 nm resulting from the formation of the carbon monoxide complex of the reduced form of the pigment. Values expressed as nanomoles of cytochrome per milligram (dry weight).

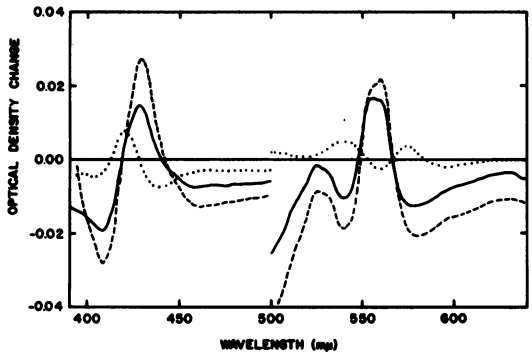

FIG. 3. *Difference spectra of glucose-grown P. putida. The substrate-reduced minus oxidized (solid line), $Na_2S_2O_4$-reduced minus oxidized (dashed line), and $Na_2S_2O_4$-reduced plus carbon monoxide minus reduced (dotted line) difference spectra were recorded essentially as described in the legend to Fig. 1, except 10 mg (dry weight) of cells per ml were used for the visible region and 2 mg (dry weight) of cells per ml were used for the Soret region of the spectrum.*

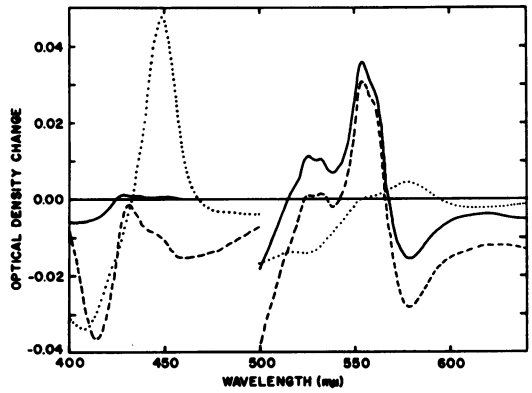

FIG. 4. *Difference spectra of camphor-grown P. putida. The substrate-reduced minus oxidized (solid line), $Na_2S_2O_4$-reduced minus oxidized (dashed line), and $Na_2S_2O_4$-reduced plus carbon monoxide minus reduced (dotted line) difference spectra were recorded as described in the legend to Fig. 1. The cell concentrations used for the visible region were 10.0 mg (dry weight) of cells per ml and for the Soret region 2.0 mg (dry weight) of cells per ml.*

results obtained by both the calculation from the spectrum of whole cells and by the extraction procedure agreed very well (0.14 nmoles per mg in both cases). These results indicate that measuring cytochrome *c* content from difference spectra in these organisms is a valid procedure.

The content of protoheme in *P. putida* doubles when the cells are grown on camphor as sole carbon source as compared to cells grown on glucose (Table 3). The amount of cytochrome *o* is sufficient to account for most of the protoheme in glucose-grown cells; however, in camphor-grown cells, the content of cytochrome *o* cannot be estimated reliably because of the large absorption bands associated with cytochrome P-450. Cytochrome *o* has been found to be associated with cell membrane fragments prepared from camphor-grown cells (J. Peterson, *unpublished data*). The content of cytochrome P-450 in glucose-grown cells is so low that it is undetectable even by the sensitive techniques employed in this study.

Included in Table 3 is the content of nonheme iron protein, as estimated by the area of the $g = 1.94$ EPR signal. The content of nonheme iron protein increases by a factor of 9 when the cells are grown on camphor as carbon source. The EPR signals of glucose- and camphor-grown *P. putida* are shown in Fig. 5. The nonheme iron protein which gives the $g = 1.94$ signal in glucose-grown cells is associated with a membrane-bound respiratory system and is not easily solubilized by sonic oscillation of the cells. The nonheme iron protein which is associated with the increase in the $g = 1.94$ signal in camphor-grown cells is readily liberated by sonic oscillation and appears to be different from the nonheme iron protein of glucose-grown cells.

DISCUSSION

The majority of the mixed function oxidases of bacteria which were purified and characterized were of the single-protein component type (8). However, there are other bacterial mixed function oxidases which require a multicomponent electron transfer system (12, 16, 24). Typical of these

TABLE 3. *Cytochrome content of P. putida*

Cytochrome	Glucose-grown	Camphor-grown
Cytochrome *c*	0.12	0.14
Protoheme	0.10	0.22
Cytochrome *o*	0.07	
Cytochrome P-450	0	0.15
Nonheme iron protein	0.02	0.18

The content of cytochrome *c*, protoheme, and cytochrome *o* were determined as described in the legend to Table 2 and in the text. The content of cytochrome P-450 was determined from the absorption change at 446 nm resulting from the formation of the carbon monoxide complex of the reduced form of the pigment. The content of nonheme iron protein was determined from the area of the integrated electron paramagnetic resonance spectrum and related to a standard sample of adrenodoxin. Values expressed as nanomoles of cytochrome per milligram (dry weight).

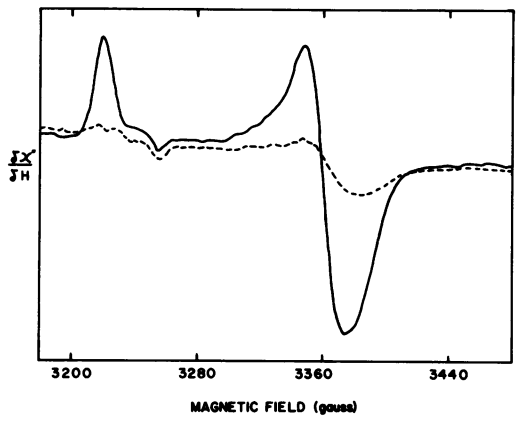

FIG. 5. *EPR spectrum of glucose- and camphor-grown P. putida. The EPR spectrum of $Na_2S_2O_4$-treated cells was recorded by using a Varian E4 EPR spectrometer equipped with a variable temperature attachment. The concentrations of the cells were adjusted to 30 mg (dry weight)/ml and the cells were treated with $Na_2S_2O_4$ to make certain the putidaredoxin was reduced. The quartz EPR tubes were selected to give equal instrument response with identical samples. The various instrument parameters were: modulation amplitude, 12.5 gauss; modulation frequency, 9.148 GHz; microwave power, 50 mw; temperature, −172 C; scan time, 8 min; time constant, 1 sec. The glucose-grown cells are denoted by the dashed line and the camphor-grown cells by the solid line.*

multicomponent mixed function oxidase systems is the ω-hydroxylase system of *P. oleovorans* which requires a NADH-dependent rubredoxin reductase, rubredoxin, and the ω-hydroxylase (16).

This paper describes the changes in cytochromes and nonheme iron proteins in *P. oleovorans* upon induction of the ω-hydroxylase system. No change was found in the content of nonheme iron protein, as estimated by the area of the EPR signal at $g = 1.94$. These results are to be expected because, although rubredoxin is a type of nonheme iron protein, it does not have an EPR signal at $g = 1.94$. Spectral evidence for the presence of rubredoxin in hexane-grown *P. oleovorans* is also lacking. The absorption band of oxidized rubredoxin at 495 nm has an ξ_M of 11.1×10^3 (17), and, therefore, a large decrease in absorption upon reduction of the rubredoxin might not be expected. The EPR signal of oxidized rubredoxin at $g = 4.3$ (17) was also not observed in hexane-grown cells, since it is very difficult to obtain an oxidized sample of bacterial cells for EPR spectroscopy.

The content of cytochrome *o* in hexane-grown *P. oleovorans* is higher by a factor of 4.5 than that in cells grown on glucose as carbon source. The spectral evidence presented in this paper indicates that *P. oleovorans*, when grown on hexane, does not contain cytochrome P-450. The problem of the nature of the functional group of the ω-hydroxylase still remains. Since the content of cytochrome *o* is markedly higher in cells grown on hexane, the possibility exists that cytochrome *o* is involved in hexane metabolism even though prior studies have shown that the ω-hydroxylation of hydrocarbons is not inhibited by carbon monoxide (18).

Similar to the *P. oleovorans* ω-hydroxylase system (24) is the ω-hydroxylase system of *P. desmolytica* grown on heptane (14). A different type of ω-hydroxylase system was found in *Corynebacterium* species (2) which were grown on octane as sole carbon source. The ω-hydroxylation of octane by extracts from *Corynebacterium* sp. was inhibited by carbon monoxide and the ω-hydroxylase fraction contained a type of cytochrome P-450 (2). Preliminary experiments with the yeast *Candida lipolytica* grown on hexadecane as sole carbon source failed to demonstrate the presence of cytochrome P-450 in these cells. From these results, it must be concluded that there are at least two types of microbial ω-hydroxylase systems.

Metabolic studies with *P. oleovorans* were presented in this paper to demonstrate that the cells used for the spectral analysis were competent to oxidize hexane. Cell-free extracts prepared from similar preparations of cells were used for the determination of the stoichiometry of the ω-hydroxylation reaction (19). The substrates, other than hexane, were chosen to determine whether the basic metabolism of the cells was altered by growth on the substrates for mixed function oxidation. The probable cause of the fourfold increase in respiration on ethanol is the induction of an alcohol dehydrogenase similar to the alcohol dehydrogenase induced in *P. aeruginosa* grown on heptane (1). The level of understanding of the metabolism of *P. oleovorans* does not permit speculation on the meaning of the other changes in respiratory ability. The cause of these metabolic changes could be due to an alteration in the permeability of the ions utilized.

Another well-characterized bacterial multicomponent mixed function oxidase system is the camphor methylene hydroxylase of *P. putida* (12). The results presented in this paper on the content of cytochromes and nonheme iron protein in *P. putida* serve as a contrast to the previously discussed ω-hydroxylase system of *P. oleovorans*. In *P. putida* there is a very large change in total content of protoheme, cytochrome P-450, and

nonheme iron protein, whereas no such changes were observed in *P. oleovorans*.

The extinction coefficient used for the cytochrome P-450 determination is higher than normally used; however, if the literature value of approximately 100 (20) were used for the calculation of the concentration of cytochrome P-450, the amount of cytochrome P-450 would be larger than the total content of protoheme. Because of the degree of uncertainty surrounding the extinction coefficient of microsomal cytochrome P-450 (10), it seems reasonable to conclude that the extinction coefficient of cytochrome P-450 in whole bacterial cells might be different from isolated cytochrome P-450. By using the higher extinction coefficient, which would tend to underestimate the content of cytochrome P-450, cytochrome P-450 is found to account for at least 40% of the total heme content of the whole cells which were grown on camphor. The cause of the larger extinction coefficient for cytochrome P-450 in whole cells does not seem to be a result of the turbidity associated with whole cell suspensions. The content of cytochrome *c* in these bacteria could be determined with equal reliability either with whole cells or by the extraction procedure. The cause of the change of the extinction coefficient of cytochrome P-450 in whole cells may be an intramolecular association of the components of the methylene hydroxylase system.

The EPR spectrum of $Na_2S_2O_4$-treated camphor-grown *P. putida* cells closely resembles the spectrum of putidaredoxin (5). At no time was an EPR signal similar to that associated with microsomal cytochrome P-450 (9) observed in these bacteria. The level of cell respiration was sufficiently high so that all of the samples were anaerobic, whether or not they were treated with $Na_2S_2O_4$; cytochrome P-450 in the reduced form has no EPR signal.

The data presented in Table 3 indicate that the ratio of nonheme iron protein to cytochrome P-450 in cells grown on camphor is close to one. This value is routinely obtained with cells harvested during late-log-phase growth. However, the ratio of nonheme iron protein to cytochrome P-450 is two in cells which are rapidly growing on camphor or in cells during the induction of the camphor metabolism system.

The components of the methylene hydroxylase system, determined for this paper, represent a significant portion of the total weight of the bacterial cell. If the molecular weights of cytochrome P-450 and putidaredoxin are assumed to be 40,000 and 16,000, respectively, these two components of the methylene hydroxylase system represent 0.9% of the total dry weight of the organism. The amount of putidaredoxin and cytochrome P-450 per kilogram (wet weight) of cells is 36 μmoles and 30 μmoles, respectively. These numbers illustrate that the concentrations of the components of the methylene hydroxylase system of *P. putida* are appreciable and any study of the kinetic interaction of these components must take these concentrations into account.

A subsequent paper will describe the kinetics of the induction of the components associated with the camphor methylene hydroxylase system of *P. putida*.

ACKNOWLEDGMENTS

The author expresses his appreciation to Ronald W. Estabrook for advice and encouragement, and to Mrs. R. S. Bailey for expert technical assistance during these investigations.

This investigation was supported by Public Health Service research grant 13366 from the National Institute of Arthritis and Metabolic Diseases.

LITERATURE CITED

1. Azoulay, E., and M. T. Heydeman. 1963. Extraction and properties of alcohol dehydrogenase from *Pseudomonas aeruginosa*. Biochim. Biophys. Acta 73:1–6.
2. Cardini, G., and P. Jurtshuk. 1968. Cytochrome P-450 involvement in the oxidation of *n*-octane by cell-free extracts of *Corynebacterium* sp. strain 7E1C. J. Biol. Chem. 243: 6070–6072.
3. Castor, L. N., and B. Chance. 1959. Photochemical determinations of the oxidases of bacteria. J. Biol. Chem. 234:1587–1592.
4. Cooper, D. Y., H. Schleyer, and O. Rosenthal. 1968. Role of cytochrome P-450 in mixed function oxidases using the reconstituted steroid 11β-hydroxylase of adrenal mitochondria as an example. Hoppe-Seyler's Z. Physiol. Chem. 349:1592–1598.
5. Cushman, D. W., R. L. Tsai, and I. C. Gunsalus. 1967. The ferroprotein component of a methylene hydroxylase. Biochem. Biophys. Res. Commun. 26:577–583.
6. Gelman, N. S., M. A. Lukoyanova, and D. N. Ostrovskii. 1967. The respiratory chain system of bacteria, p. 71–140. *In* G. B. Pinchot (translation ed.), Respiration and phosphorylation of bacteria. Plenum Press, New York.
7. Gunsalus, I. C. 1968. A soluble methylene hydroxylase system: structure and role of cytochrome P-450 and iron-sulfur protein components. Hoppe-Seyler's Z. Physiol. Chem. 349:1610–1613.
8. Hayaishi, O. 1969. Enzymic hydroxylation. Annu. Rev. Biochem. 38:21–44.
9. Hashimoto, Y., T. Yamano, and H. S. Mason. 1962. An electron spin resonance study of microsomal electron transport. J. Biol. Chem. 237:3843–3844.
10. Hildebrandt, A., H. Remmer, and R. W. Estabrook. 1968. Cytochrome P-450 of liver microsomes—one pigment or many. Biochem. Biophys. Res. Commun. 30:607–612.
11. Imai, K., A. Asano, and R. Sato. 1967. Oxidative phosphorylation in *Micrococcus denitrificans*. I. Preparation and properties of phosphorylating membrane fragments. Biochim. Biophys. Acta 143:462–476.
12. Katagiri, M., B. N. Ganguli, and I. C. Gunsalus. 1968. A soluble cytochrome P-450 functional in methylene hydroxylation. J. Biol. Chem. 243:3543–3546.
13. Kusunose, M., E. Kusunose, and M. J. Coon. 1964. Enzymatic ω-oxidation of fatty acids. I. Products of octanoate, decanoate and laurate oxidation. J. Biol. Chem. 239:1374–1380.
14. Kusunose, M., J. Matsumoto, K. Ichihara, E. Kusunose, and J. Nozaka. 1967. Requirement of three proteins for hydrocarbon oxidation. J. Biochem. 61:665–667.

15. Omura, T., R. Sato, D. Y. Cooper, O. Rosenthal, and R. W. Estabrook. 1965. Function of cytochrome P-450 of microsomes. Fed. Proc. 24:1181–1189.
16. Peterson, J. A., D. Basu, and M. J. Coon. 1966. Enzymatic ω-oxidation. I. Electron carriers in fatty acid and hydrocarbon hydroxylation. J. Biol. Chem. 241:5162–5164.
17. Peterson, J. A., and M. J. Coon. 1968. Enzymatic ω-oxidation. III. Purification and properties of rubredoxin, a component of the ω-hydroxylation system of *Pseudomonas oleovorans*. J. Biol. Chem. 243:329–334.
18. Peterson, J. A., M. Kusunose, E. Kusunose, and M. J. Coon. 1967. Enzymatic ω-oxidation. II. Function of rubredoxin as the electron carrier in ω-hydroxylation. J. Biol. Chem. 242:4334–4340.
19. Peterson, J. A., E. J. McKenna, R. W. Estabrook, and M. J. Coon. 1969. Enzymic ω-oxidation: stoichiometry of ω-oxidation of fatty acids. Arch. Biochem. Biophys. 131:245–252.
20. Rieske, J. S. 1967. The quantitative determination of mitochondrial hemoproteins, p. 488–493. *In* R. W. Estabrook and M. E. Pullman (ed.), Methods in enzymology, vol. 10. Academic Press Inc., New York.
21. Scholes, P. B., and L. Smith. 1968. Composition and properties of the membrane-bound respiratory chain system of *Micrococcus denitrificans*. Biochim. Biophys. Acta 153: 363–375.
22. Smith, L. 1968. The respiratory chain system of bacteria, p. 55–122. *In* T. P. Singer (ed.), Biological oxidations. Interscience, New York.
23. Webster, D. A., and D. P. Hackett. 1966. The purification and properties of cytochrome *o* from *Vitreoscilla*. J. Biol. Chem. 241:3308–3315.
24. Yu, C. A., and I. C. Gunsalus. 1969. Monooxygenases. VII. Camphor ketolactonase I and the role of three protein components. J. Biol. Chem. 244:6149–6152.