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ABSTRACT

Mark Charles Gunderson. PURIFICATION AND SPECTRAL CHARACTERISTICS OF CYTOCHROME C-551 IN A PHTHALATE METABOLIZING BACTERIUM. (Under the direction of Takeru Ito) Department of Biology, July, 1979.

Routine inspection of the harvested bacterial cells from a phthalate metabolizing bacterium showed a reddish tint which was thought to be due to the presence of cytochromes. This report describes purification and characterization of cytochrome c from this organism.

Nutrient broth was chosen as the growth medium since it produced the largest quantities of cytochrome c-551 per gram dry weight of bacteria. It was found that nutrition plays a role in the relative concentrations cytochrome c-551 and cytochrome b-558 in the experimental organism.

The French Press was found to be very efficient in liberating the cytochrome c-551 from the bacterial cells. The extracted c-551 was further purified with ammonium sulfate fractionation and ion-exchange techniques with SP-Sephadex. The major purification resulted from isoelectric focusing which resulted in 250 fold purification over the original cell free extract. Gel electrophoresis confirmed the purity of the final product was very high.

The cytochrome c-551 had reduced maxima at 551, 522, and 416 nm. The oxidized peak was at 408 nm. The protein appeared to be a typical cytochrome c similar to many of the prokaryotic c-type cytochromes and mitochondrial cytochrome c.

Evidence of electron transport capabilities of the highly purified

enzyme indicated that the isolation procedures had not damaged the enzymatic functions of the cytochrome c-551.

PURIFICATION AND SPECTRAL CHARACTERISTICS OF CYTOCHROME C-551 IN A PHTHALATE METABOLIZING BACTERIUM

A Thesis

Presented to

the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

by

Mark C. Gunderson

October 1978

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IN A PHTHALATE METABOLIZING BACTERIUM

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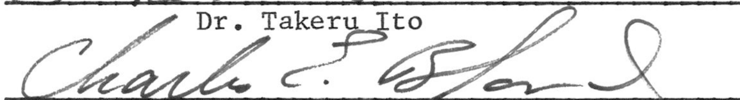

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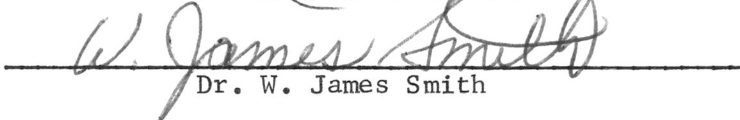

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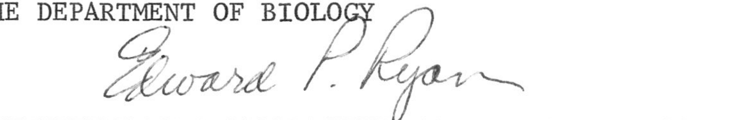

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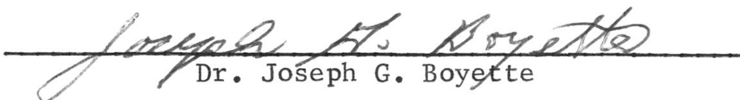

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INTRODUCTION

During the course of a preliminary study involving phthalate metabolism in soil bacteria, it was noticed that harvested cells, of what was thought to a strain of *Pseudomonas*, appeared reddish. It was speculated that the reddish tint was due to the presence of cytochromes. Bacterial c-type cytochromes have been at least partially purified from a wide range of organisms. The predominant impression is one of overwhelming diversity. Regarding spectral properties, isoelectric point, molecular weight, number of heme groups per molecule, standard oxidation-reduction potential, bacterial c-type cytochromes vary greatly. These properties, along with corresponding bacterial species, have been extensively listed (Dickerson and Timkovich, 1975). All of the evidence has shown that the bacterial c-type cytochrome functions in electron transport chains with coupled energy saving processes. A study was proposed to attempt to purify cytochrome c from this organism and to determine the spectral characteristics of this protein. The cytochrome c will be compared with other known bacterial c-type cytochromes, and the biological activity of the protein will be demonstrated.

REVIEW OF LITERATURE

The following account of discovery and rediscovery of cytochromes as described by Keilin (1966) is well documented. The accumulation of knowledge on cytochromes as respiratory pigments began with the spectroscopic observations of these pigments by MacMunn in 1886. From a variety of tissues, MacMunn found a characteristic absorption spectrum for pigments he named myohematin and histohematin. The former name was given to the pigment found in muscle and the latter to an identical pigment found in other tissues. Destructive criticism of MacMunn's work by leading scientists of his day delayed the development of research on cytochromes for 40 years. It remained for Keilin in 1925 to show that MacMunn's observations were fundamentally correct. Keilin, recognizing the significance of these pigments in biological oxidations, renamed them cytochromes (meaning cell pigments) and grouped them into three major classes (a, b, and c) depending on the position of the absorption bands. Keilin recognized also that these compounds were not simple hematins, such as hemoglobin and myoglobin, but rather hemoproteins of a class of compounds now known as ferro- and ferricytochromes. He was aware also that the change in iron valency of these compounds was connected to their respiratory function as electron carriers.

The respiratory cytochrome c found in the mitochondria of all eukaryotes is a basic protein with one heme c and 103 to 113 amino acids in a single polypeptide chain. It is small, having a molecular weight of 12,500, and is easily extracted from ruptured mitochondria. This is in contrast to other protein components of the respiratory chain, which

are generally larger and membrane bound.

Bodo (1955) was the first to crystallize cytochrome c from King Penguin muscle. By very similar methods, crystalline cytochrome c has been obtained from a variety of sources such as baker's yeast (Hagihara _et al_., 1956), ox heart (Kuby _et al_., 1956), horse heart (Nozaki _et al_., 1975), fish heart (Hagihara _et al_., 1957) and wheat germ (Hagihara _et al_., 1958).

Because of its universality and ease of extraction and purification from eukaryotes, cytochrome c has been the subject of amino acid sequence determinations in more different species than any other protein. Dickerson and Timkovich (1975) have compared the sequences of 67 c-cytochromes from species of plants, animals, and microorganisms. The results of this study have been the basis for an extensive evaluation of eukaryotic evolution. In this study, a phylogenetic tree has been established (Dickerson, 1972) solely on the basis of amino acid differences in various c-cytochromes. Relationships reflected in this phylogenetic tree agree well with those determined previously from comparative anatomy and the fossil record.

Eukaryotic c-cytochromes from several species have been studied also by x-ray crystallographic methods (Dickerson and Timkovich, 1975). The structural homologies of these proteins are striking. In general, c-cytochromes from eukaryotes are all similar in size, three-dimensional structure, and oxidation-reduction potential. The basic structure and function of the molecule has remained essentially unchanged since eukaryotes have evolved.

Although there has been much less work done on bacterial cytochromes,

it appears that they are much more diverse than eukaryotic ones (Dickerson and Timkovich, 1975). Difficulties associated with production of large quantities of cells and mechanical breakage of the small bacterial cell, which often has a tough cell wall, have led to the limited knowledge of bacterial cytochromes. It is also characteristic that the cytochromes of bacteria are more firmly membrane bound. However, several of the more robust bacterial cytochromes have been purified and their primary structure revealed.

Cytochromes c are universal among bacterial species, with the exception of the strictly anaerobic fermenters such as _Clostridia_ and some anaerobes such as _Streptococci_ (Dickerson and Timkovich, 1975). Even though bacterial c-cytochromes vary greatly in spectral qualities, size, heme content, and redox potential, several broadly defined classes are recognized (Dickerson and Timkovich, 1975). The most common class includes the proteins similar in size and in high redox potential to the typical eukaryotic cytochrome c. There are two subclasses of this class, the first being a low molecular weight subclass (8,000) includes _Pseudomonas aeruginosa_ c-551. The other subclass typified by the purple non-sulfur bacteria _Rhodospirillium rubrum_ c_2, has a molecular weight of 12,500. The second is the c' class which consists of tightly bound dimers of cytochrome c. It has been isolated from purple photosynthetic bacteria and from some denitrifying species and is characterized by having a split alpha absorption band. The large cytochromes c with bound flavins such as _Chromatium_ c-552 are also in a class by themselves. The fourth class consists of the low potential multiheme cytochromes c_3 from various species of _Desulfovibrio_ which are anaerobic sulfate reducers. In summary,

bacterial cytochromes c are found in all species which contain electron transfer chains in which oxidation is coupled to phosphorylation.

In contrast to mitochondrial cytochrome c of eukaryotes which appears to have remained essentially unchanged in structure through evolution and is thus considered one of the most evolutionarily conservative proteins (Dickerson and Timkovich, 1975), bacterial cytochromes c are much more diverse. X-ray crystallographic studies along with sequence determinations have shown that the large group of bacterial cytochromes c which are similar in size and redox potential to eukaryotic cytochrome c may have all descended from a common ancestral protein (Salemme, 1977). Nevertheless, there is evidence for considerable structural diversification among the prokaryotic members of this class (Dickerson and Timkovich, 1975). The construction of a detailed phylogenetic tree in prokaryotes is much more difficult than with eukaryotes. Exchange of genetic material by viral infection and transduction of large segments of the bacterial genome possibly has blurred the true history of the present-day bacteria beyond recall. Dickerson *et al.*, (1976) has proposed that bacterial and eukaryotic oxygen respiration arose from photosynthetic and respiratory electron transport chains, in purple non-sulfur bacteria, by loss of the photosynthetic capabilities. At present, however, the diverse spectrum of bacterial cytochromes c remains unclear in terms of evolutionary homologies. As more sequence determination and structural analysis of the many varieties of bacterial cytochromes c are known, the evolutionary homologies may be clarified.

Purification of mitochondrial cytochrome c has been relatively easy because of its ease of extraction and the fact that large quantities of

tissues can easily be obtained. Due to similarities in amino acid sequence and structure in these proteins, the method of purification has remained essentially the same. In most cases, cytochrome c has been purified from the crude to the purest state by chromatography on a weak cation ion-exchanger resin (Horio *et al*., 1960).

Readily soluble cytochrome c_2 has been purified from the purple non-sulfur bacterium *Rhodospirillium rubrum* (Dickerson and Timkovich, 1975). This cytochrome was easily released by lyophilization or sonification of the bacteria. Amberlite CG-50 was used to purify the protein. Horio *et al*., (1960) prepared crystalline *Pseudomonas aeruginosa* c-551. This organism produces large amounts of c-551 when grown anaerobically in a medium containing nitrate as a terminal electron acceptor. This method was later modified by Ambler (1963), with use of carboxymethyl-cellulose and diethylaminoethyl-cellulose (DEAE-cellulose) ion-exchange resins. Ambler and Wynn (1973) later used this method of purification for subsequent studies of the amino acid sequence of three species of *Psuedomonas*. Cytochrome c-550 and c-554 have been purified from *Bacillus subtillus* (Miki and Okinuki, 1969) with DEAE-cellulose. Cytochrome c-552 has been purified from an extreme thermophile, *Thermus thermophillus* (Hon-ami and Oshima, 1977) with Amberlite CG-50 and Sepha dex G-75. Cytochrome c-552 has been purified also from a sulfur-oxidizing bacterium, *Thiobacillus thioxidans* by a similar procedure (Takakuwa, 1975). Cytochrome c-551.5 has been purified from *Desulfomonas acetoxidans*, an anaerobic, sulfur-reducing bacterium (Probst *et al*., 1977).

In general, there is no single method that is applicable for purification of bacterial cytochromes c. Each particular cytochrome c

possesses its own characteristics which will determine the ultimate method of purification. Some of the various cytochromes c from some prokaryotes are not very soluble and purification is extremely difficult. Disruption of the bacterial cell wall is sometimes difficult. For example, certain species such as Staphylococcus possess extremely tough cell walls and disruption is very difficult (Dickerson and Timkovich, 1975). Another major problem is that most bacterial species produce only minute quantities of cytochrome c. Bacterial cytochromes c have usually been studied in those species which produce large amounts of cytochrome c. Once the cytochrome c has been released from the cell, further purification is, theoretically, a routine matter. Experiments involving ammonium sulfate fractionation, ion-exchange procedures, and electrophoretic studies are usually tried in order to determine a purification scheme. Thus, each different bacterium, with its own unique cytochrome c, presents a challenge to the researcher attempting purification of the protein.

MATERIALS AND METHODS

Sterile saline solution

Sodium chloride (0.9%) was dispensed into 13 x 150 mm test tubes in 5.4 ml portions. The tubes were then autoclaved and used for sterile transfer of the bacteria (Seaman, 1962).

Bacterial salt solution

The following salt solution was made for addition to the enrichment culture medium: 20.0 g $MgSO_4 \cdot 7 H_2O$, 0.2 g $FeSO_4 \cdot 7 H_2O$, 0.1 g $ZnSO_4 \cdot 7 H_2O$, 0.02 g $Mncl_2 \cdot 4 H_2O$, and 0.002 g $CuSO_4 \cdot 5 H_2O$, 100 ml of 1 N HCl, and 900 ml deionized water. Ten ml portions were added to each liter of enrichment culture medium prepared.

Enrichment culture medium

A culture medium containing potassium hydrogen phthalate as the sole carbon source was prepared to isolate a soil bacterium that could metabolize this compound. The medium was composed of 1.0 g of NH_4Cl, 1.5 g of K_2HPO_4, 0.5 g of NaH_2PO_4, 0.1 g of yeast extract, 0.5 g of potassium hydrogen phthalate, 200 ml of tap water, 10 ml of the bacterial salt solution, and 790 ml of deionized water. The pH was adjusted to 7.0 with 1 N KOH and the 1 liter medium autoclaved.

Agar plates and agar slants

To 200 ml of the enrichment culture medium, 3.0 g of Difco agar was added and the mixture was dissolved by gentle heating. The solution was then autoclaved. The sterile, hot agar solution was then added aseptically into sterile petri dishes in approximately 20 ml portions.

Agar slants were prepared by 10 ml additions of the hot agar solution into sterile, 16 x 150 mm screw cap tubes. The tubes, while hot, were placed at an angle of approximately 20° and were left to cool. The cooled tubes were then stored until used.

Isolation of bacterial strain

Approximately 0.5 g of a soil sample from a Pitt county forest was added to a test tube containing sterile saline solution. After a period of 6 hours, 0.6 ml of the slightly turbid solution was aseptically transferred into three test tubes containing 6.0 ml of the sterile enrichment culture medium. The tubes were incubated overnight at room temperature. The appearance of turbidity in the tubes indicated bacterial growth, and 0.1 ml aliquots of the liquid medium were aseptically transferred into another three tubes containing fresh culture medium. These tubes were incubated at room temperature overnight.

The test tube containing the most vigorous growth was selected to make a serial ten-fold dilution. Eight test tubes, each containing 5.4 ml of sterile saline solution were used to make the dilutions. From the most actively growing tube, 0.6 ml was transferred into the first saline tube with a sterile pipette and suspended uniformly. From the first saline tube, 0.6 ml was aseptically transferred into the second saline tube, and from the second into the third and so forth. These serial ten-fold transfers resulted in a one hundred million fold dilution in the last saline tube of the original bacterial suspension. From each saline test tube, 0.1 ml was transferred aseptically into a corresponding agar plate and spread uniformly. Eight such plates were prepared and they were stored at room temperature for 72 hours. The

first four plates exhibited intense bacterial growth. The more diluted plates showed small numbers of large, round, discrete colonies of bacteria. The size and shape of certain colonies indicated that each arose from a single bacterial cell. The most vigorously growing colony was selected as the experimental organism. The colony was aseptically transferred into a agar slant and incubated for 24 hours. From this tube the entire dilution process and corresponding growth on agar plates was repeated to insure the purity of the culture. The pure bacterial strain was streaked aseptically into agar slants and incubated overnight at room temperature. The stock cultures were then placed in a refrigerator for storage.

<u>Nutrient agar slants</u>

These were prepared by suspending 31 grams of 1.5% Nutrient Agar (BBL) in a liter of deionized water and heating gently to dissolve the agar. The solution was then autoclaved and aseptically dispersed while still warm, in 10 ml portions, into sterile 16 x 150 mm screw cap tubes and tilted at an angle of 20°. The cooled slants were used also for stock cultures of the phthalate utilizing bacterium.

<u>Growth media used</u>

Six different media were made to determine which one produced the most number of bacterial cells and also which media produced the largest quantity of cytochrome c-551. The media were prepared as follows:

1. Phthalate Medium: 1.0 g of NH_4Cl, 1.5 g of K_2HPO_4, 0.5 g of NaH_2PO_4, 0.5 g of yeast extract (Difco), 1.0 g of potassium hydrogen phthalate, 10 ml of the bacterial salt solution, 200 ml of tap water,

and 790 ml of deionized water to make a 1 liter solution. The dissolved solution was adjusted to pH 7.0 with 6 N KOH and autoclaved in a cotton-plugged 2 liter flask.

2. Phthalate-Glutamate Medium: This medium was prepared identical to the phthalate medium with the difference being the addition of 5.0 g of sodium glutamate.

3. Phthalate-Glutamate-Nitrate Medium: This medium was prepared identical to the phthalate-glutamate medium with the difference being the addition of 5.0 g of $NaNO_3$.

4. Nutrient Broth Medium: 8.0 g of Nutrient Broth (BBL) was dissolved per liter of medium desired with deionized water. There was no pH adjustment necessary. The prepared medium was then autoclaved in a 2 liter flask.

5. Nutrient Broth-Nitrate Medium: This was prepared identical to the nutrient broth medium with the only difference being the addition of 5.0 g of $NaNO_3$.

6. Citrate-Nitrate Medium: This was prepared by the addition of 5.0 g of sodium citrate, 5.0 g of $NaNO_3$, 1.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7 H_2O$, and 4.0 g of yeast extract, 200 ml of tap water, and 800 ml of deionized water. The dissolved medium was adjusted to pH 7.0 with 6 N KOH and autoclaved.

Small scale harvest of bacteria

For aerobic growth, one liter portions of the bacterial growth medium in a 2 liter flask were innoculated aseptically with the stock culture. The flasks were then put on a New Brunswick Company Gyrotory Shaker at room temperature with sufficient agitation to provide even

oxygen distribution throughout the culture medium. When anaerobic conditions were desired, the innoculated flasks were left standing at room temperature with no shaking.

Aerobic cultures were harvested, usually after 24 hours, by centrifugation in 250 ml plastic bottles using a Sorvall GSA rotor in a Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge at 10,000 x g. The harvested cells were kept at ice temperature and were washed twice with 0.01 M potassium phosphate buffer, pH 7.0 and used immediatly or kept on ice until the next day. Anaerobic cultures were harvested after 72 hours growth as more time was needed to obtain a maximum number of cells. They were harvested in a similar manner as the aerobically grown cells.

Large scale harvest of the bacteria

Thirty-six liters of nutrient broth medium was prepared and dispensed in three liter portions into 4 liter flasks. The flasks were then plugged with cotton and autoclaved. The cooled flasks were innoculated and put on a New Brunswick Scientific Company Controlled Environment Shaker at 150 rpm and 37° C. The cultures were incubated for 24 hours. The cells were harvested as before and washed twice with buffer. The twice washed pellet was then frozen rapidly in a solid CO_2/acetone mixture and lyophilized on a Virtis Model 10 Lyophilizer System. The lyophilized cells were stored in a freezer at -10° C. for further cytochrome c-551 purification studies.

Determination of dry weight of the bacterial cells

The dry weight of the bacterial cells was determined by the method

of Ito (unpublished). The method is as follows: Cell suspensions were diluted appropriately with deionized water, usually 1:100 or 1:200, and the absorbance at 540 nm was recorded with a Beckman DB Spectrophotometer. If the absorbance was more than 0.5, the cell suspension was diluted further. The concentration of the cells in the diluted suspension in mg dry weight cells/ml suspension was calculated by dividing the absorbance by 3.68. The dry weight of the cells in mg/ml of the original suspension was calculated by multiplying the dilution factor (100 or 200) times the ratio obtained from the diluted suspension.

Cytochrome c-551 assay in whole cells

The concentration of cytochrome c-551 was determined by the method of Kodama and Shidara (1969). This method is as follows: The cells were measured spectrophotometrically on a Cary 17 D Spectrophotometer using difference spectroscopy. A portion of the cell suspension was oxidized with a few grains of solid $K_3Fe(CN)_6$ and placed in the reference side. Another portion, in a similar cuvette, was reduced with an excess of a few grains of sodium dithionite. This was placed in the sample side. Then, the difference spectrum was made by a scan from 600 nm to 500 nm and recording the scan on chart paper. The differences in absorbancy at the following pairs of wavelengths were taken from the difference spectrum. The pairs are as follows: 551 nm - 538 nm for c-551 and 558 nm - 575 nm for cytochrome b-558. A double equation was used to calculate the various cytochrome concentrations since absorbancy of a cytochrome includes interference from the other. The double equation is as follows:

$$A_1 = 13.5\ X_c + 5.5\ X_b$$

$$A_2 = 6.5\ X_c + 17.5\ X_b$$

where X_c = concentration of c-551 in mM and X_b = the same for b-558: A_1 = the difference in absorbancies at 551 nm and 538 nm and A_2 = the difference at 558 nm and 575 nm. In this manner, the respective quantities of cytochromes c-551 and cytochrome b-558 were calculated in whole cells.

Cytochrome c-551 assay in soluble fractions

Absorption spectra were measured on a Cary 17D Spectrophotometer using cuvettes of 1 cm light path and 1.5 ml capacity. The amount of cytochrome c-551 was determined by the method of Ambler (1963). The sample in buffer was adjusted to pH 7.0. The sample was first oxidized by the addition of 50 microliters of 1 mM $K_3Fe(CN)_6$ and a spectrum recorded of a 600 nm to 500 nm scan. The sample was then reduced with an excess of sodium dithionite and the spectrum recorded as before. The difference in absorbancy between the oxidized and reduced spectrum at the position of the alpha band, 551 nm, was recorded. The difference in molar extinction coefficients at this wavelength was assumed to be 20 x 10^3 mole $^{-1}$ cm $^{-1}$. From this value, quantitation of cytochrome c-551 in solution was made.

Determination of protein concentration

Protein concentrations in the various purification stages were determined by the method of Layne (1957). This method is based on UV absorption at 260 nm and 280 nm. Proteins absorb at 280 nm. The 260 nm region is where nucleic acids absorb. This method takes interference

effects into account in determining protein levels. The solutions to be measured are first diluted appropriately and the absorbance at 260 nm and 280 nm recorded. The protein concentration was then determined in the following manner:

1. Calculate the ratio of optical density at 280 nm/ 260 nm.
2. Use the preceding value to determine the corresponding F value (Layne, 1957).
3. Calculate the protein concentration from the equation: Absorbance_{280} times (F) times (1/d) = mg/ml protein, where d = the diameter of the light path which is 1 cm.

Preparation of SP-Sephadex

30.0 grams of dry SP-Sephadex (Sigma) resin were swollen for 24 hours in deionized water. The swollen resin was then placed in 0.5 M NaOH for 30 minutes and then repeatedly washed with deionized water until the pH of the resin reached neutrality. The resin was then equilibrated by the addition and repeated washing with the respective starting buffer.

Preparation of DEAE-Cellulose

50.0 grams of DE52 (Whatman) was repeatedly washed with deionized water until neutrality was reached. The DEAE-cellulose was then placed in a vacuum apparatus and degassed after placing the ion-exchanger in 0.5 M potassium phosphate, pH 4.0. The slurry was then titrated with 0.5 M potassium phosphate, pH 9.3 until the pH reached 7.4. The ion-exchanger was then equilibrated by repeated washings with the starting buffer, .01 M potassium phosphate buffer, pH 7.4. During the washings,

the fine particles were removed by decanting.

Preparation of the cell-free extract

60 grams of lyophilized bacterial cells were homogenized with approximately 250 ml of .01 M phosphate buffer, pH 7.0. The uniform suspension was run through a pre-chilled Aminco French Pressure Cell connected to a Carver Laboratory Press. 40 ml portions of the bacterial suspension were placed into the French press and the pressure was maintained between 15,000 and 20,000 pounds/square inch. The difference in pressure broke the cell walls of the bacteria as they left the pressurized cell and returned to atmospheric pressure. The exit rate was kept at approximately 15 drops per minute. The solution was collected in a 500 ml beaker and was highly viscous due to the release of DNA by the broken cells. When all the bacterial suspension had been passed through the French press, Deoxyribonuclease I (Sigma) was added from the tip of a spatula. The solution was allowed to stir until the viscosity disappeared. The solution was then quickly cooled in an ice/salt mixture on a magnetic stirrer to 5° C. Unbroken cells and large particulate material were removed by centrifugation in 250 ml plastic bottles for 30 minutes at 25,000 x g in a refrigerated centrifuge. The brown supernatant was decanted and put on ice. The precipitate was resuspended in buffer and run through the French press again. This time no further deoxyribonuclease was added. The solution was then centrifuged as before and the supernatant was pooled with the previous. The precipitate was run through a third extraction procedure and the resulting supernatant was again pooled with the previous two. Approximately 400 ml of cell free extract was obtained and this was then

dialyzed overnight against 6 liters of cold deionized water in a cold room to remove small molecular weight compounds and excess buffer salts.

Ammonium sulfate fractionation

The dialyzed cell free extract was brought to 30% saturation of ammonium sulfate by the slow addition, while stirring, of powdered, solid, ultra-pure ammonium sulfate (Schwarz/Mann). The solution was allowed to stir for 30 minutes at 0° C. The solution was then centrifuged for 30 minutes at 25,000 x g. The supernatant was then brought to 80% saturation and allowed to stir as before. The precipitated material, which contained almost all of the cytochrome c-551, was resuspended in a minimal volume of .01 M phosphate buffer, pH 7.0, and dialyzed overnight against three changes of deionized water to remove the ammonium sulfate. The dialyzed solution was lyophilized.

Batch extraction with SP-Sephadex

The lyophilized powder from the 30-80% ammonium sulfate fraction was resuspended in a minimal volume of either .05 N ammomiun acetate, pH 3.9, or .01 M potassium phosphate, pH 3.9. The solution was adjusted, if necessary, to pH 3.9 by the addition of 50% acetic acid or 85% phosphoric acid respectively. The solution was then allowed to stir for 30 minutes at 0° C. The solution was centrifuged at 25,000 x g to remove considerable amounts of protein that was precipipated at this low pH. The following procedures were done in a Unitherm cold box at 5° C. The orangish supernatant was decanted and poured into a 600 ml beaker containing 50 ml of SP-Sephadex equilibrated with the starting buffer, either .05 N ammonium acetate, pH 3.9 or .01 M phosphate buf-

fer, pH 3.9. The mixture was stirred and the resin was allowed to settle. Most of the colored material was adsorbed to the resin. The supernatant was decanted into a second 600 ml beaker and the adsorption process repeated. This process was repeated until all of the reddish colored material had been bound to the resin. The slightly yellowish unbound material was discarded. The resin was poured into a 2.5 x 30 cm chromatography column and the resin was allowed to settle for 15 minutes. The starting buffer was run through the column until the eluted liquid turned clear. The bound material was released from the resin by the addition of either .05 M Tris, pH 10.0 or phosphate buffer, pH 10.0. The orangish eluted solution was dialyzed overnight against deionized water and lyophilized the following day.

Gradient chromatography

Gradient chromatography was attempted early in the research with DEAE-cellulose. 50 ml of the ion-exchanger was poured into an 1 x 30 cm chromatographic tube and allowed to settle. A protein sample containing cytochrome c-551 was placed at the top of the column and allowed to adhere. A chromatogram was developed with a linear gradient between .01 M phosphate buffer, pH 7.4 and the same buffer containing 2.0 M NaCl. The eluted proteins were collected with a fraction collector and the fractions were anaylyzed for protein and cytochrome c-551.

The final purification procedure contained the following procedure: The lyophilized batch-extracted powder was dissolved in a minimal volume of .01 M phosphate buffer, pH 3.9. The solution was adjusted to pH 3.9 with 6 N HCl. This solution was carefully placed on the top of

a 1 x 30 cm chromatographic column containing 60 ml of SP-Sephadex equilibrated with the starting buffer. The solution was then allowed to enter the bed of the column and a sharp reddish band was formed at the top of the column. The chromatogram was developed with a linear gradient between 250 ml of the starting buffer and 250 ml of the same buffer containing 2.0 M NaCl. A large, reddish, diffuse band was collected, dialyzed, and lyophilized. This large fraction contained all of the measurable cytochrome c-551.

Preparative isoelectric focusing

Preparative isoelectric focusing was attempted as a further purification step with an LKB Model 8101 system utilizing a 110 ml column. The following solutions were prepared:

(1) Acid Anode Solution: 0.1 ml 6 N H_2SO_4, 12 g sucrose, and 12.0 ml of deionized water.

(2) Basic Cathode Solution: 0.1 g NaOH dissolved in 10.0 ml of deionized water.

(3) Carrier Ampholytes: 1.5 ml of Biolyte 5/7 (Biorad Laboratories) was mixed with 1.0 ml Biolyte 3/10.

(4) Light Solution: 1/4 of the carrier ampholytes were dissolved in deionized water to a volume of 60 ml.

(5) Dense Solution: 3/4 of the carrier ampholytes were dissolved in deionized water to 42 ml. 28 g of sucrose was then added and dissolved in the solution.

(6) Sucrose Density Gradient Tubes: Twenty-four tubes were labeled. 4.6 ml of the dense solution was pipetted into the first tube. 4.4 ml

of the dense solution and 0.2 ml of the light solution was pipetted into the second tube. 4.2 ml of dense solution and 0.4 ml of light solution went into the third tube. This was continued until the 24th tube contained 4.6 ml of the light solution. The lyophilized sample was dissolved in tube number 15. The tubes were thoroughly mixed.

The following was pumped into the inner tube of the 110 ml Ampholine column with a polystaltic pump at a rate of 4.0 ml per minute:

(1) 10.0 ml of the acid anode solution.

(2) The sucrose density gradient tubes in numerical order.

(3) 10.0 ml of the basic cathode solution.

In this manner a preparative isoelectric focusing column was prepared with a sucrose gradient with 1% carrier ampholytes in an 110 ml column. The carrier ampholytes set up a pH gradient from 3.0 to 10.0 from anode to cathode. 60% of the carriers were chosen in the pH 5.0 to 7.0 range. This enabled good resolution of cytochrome c-551 with its isoelectric point of 6.1.

The column was connected to a Searle Model 3-1155 Power Source with an initial voltage of 500 volts. The power was not allowed to exceed 3.0 watts at any time during the experiment. The voltage was raised to 900 volts at the end of the experiment when the conductance of the column had dropped. At the end of 72 hours, the current through the column was constant indicating the proteins had migrated to their respective isoelectric points and the experiment was terminated. The column had been cooled during the experiment by a circulating water bath maintained at 5°C. The column was drained with a polystaltic pump at a rate of 2.0 ml per minute and 1.0 ml fractions were collected with an LKB Fraction

Collector. The tubes were assayed for cytochrome c-551. The tubes containing cytochromes were dialyzed to remove sucrose and carrier ampholytes and then lyophilized.

Polyacrylamide gel isoelectric focusing

Polyacrylamide gel isoelectric focusing was performed to determine the isoelectric point of cytochrome c-551 from the experimental organism. The gels were prepared as follows: 6.25 ml of monomer stock solution containing 4 M acrylamide and 50 mM Bis (N-N'-Methylenebisacrylamide) was added to a 25 ml volumetric flask. 1.25 g of reagent grade glycerol was added. 1.25 ml of Biolyte 3/10 (Biorad Laboratories) was added to the flask. The solution was mixed and 0.5 ml of a .50 mM solution of riboflavin 5-phosphate was added and the flask was filled to the line with deionized water. Approximately 2.5 ml of this solution was pipetted into 5 mm inside diameter x 125 mm glass tubes. The filled tubes were placed in front of a polymerizing light source for 10 minutes and left to stand overnight. The next day, the gels were placed in a Canalco bath assembly. The lower reservoir was filled with 0.02 N $Ca(OH)_2$, 0.04 N NaOH electrolyte solution. A magnetic stir bar was placed here and the bath assembly was placed on a magnetic stirrer. The gels were placed in the assembly with both respective ends of the gel in contact with the upper and lower reservoirs. The samples were applied to the top of the gels as 25% sucrose solutions. The samples were overlayered with 100 microliters of 20% sucrose and 100 microliters of 10% sucrose solutions. The upper ends of the tubes were then filled with the upper electrolyte, .06 N H_2SO_4. The upper electrolyte was then poured into the

upper reservoir until the tops of the gels were covered. The anode was connected to the upper reservoir and the cathode to the lower reservoir. A DC potential of 200 volts was applied to the gels for 20 hours. The Biolyte 3/10 will set up a pH gradient in the gel under the influence of the electric current and different proteins will migrate to their respective isoelectric points at that particular point in the gels. The gels were scanned at 410 and 551 nm on a Beckman DU Spectrophotometer after the experiment to detect spots on the gels where cytochrome c-551 was located. Blank gels were cut into 1 cm pieces and placed into test tubes containing 0.5 ml of deionized water for 24 hours. The ampholytes diffused out of the gels and the pH of each respective centimeter of gel was recorded. A graph of pH versus centimeter of gel was plotted. In this manner, the position of the cytochrome c-551 band identified from the absorbance scan at 410 and 551 nm was correlated with the pH at that point of the gel which corresponds to the isoelectric point of the enzyme. The cytochrome band, which was identified by the absorbance scans, as well as by visual inspection of the gels, was cut out and allowed to diffuse out of the gel into 0.5 ml of deionized water. A spectrum of the solution was taken.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed to determine the degree of purity of various fractions. The gels were prepared in the following manner: 6.5 ml of a solution containing .25 N HCl, 1.5 M Tris, 10.0 mM Temed (N, N, N', N'-tetramethylethylenediamine) was mixed with 6.5 ml of a solution containing 4 M acrylamide (Canalco) and 50 nM Bis (N, N'-

Methylenebisacrylamide). This mixture was then degassed under vacuum. The solution was cooled and mixed with 13.0 ml of a cooled 6 mM ammonium persulfate solution which acts as the catalyst to initiate polymerization. The solution was quickly pipetted into 0.5 x 12.5 cm glass tubes and left overnight to polymerize. The final concentration of the gels were 7% acrylamide. The polymerized gels were placed into a Canalco Model 1200 Bath Assembly. A 25 mM Tris, 200 mM glycine buffer, pH 8.3 was poured into the upper and lower chambers of the bath assembly which contains the electrodes which will set up the electric current through the gels. Samples were dissolved in 100 microliters of the electrode buffer. 50 microliters of .005% bromophenol blue was added as a tracking dye. A few crystals of sucrose and sodium dithionite were also added to the sample solutions. 50 microliters of sample was placed at the top of each gel with a Pasteur pipette. A current of 2.5 milliamps per gel from a Searle Model 3-1155 Power Source was maintained for 3 to 6 hours until the tracking dye had reached the ends of the gels. The gels were cooled by a water bath maintained at 0° C. circulating between the electrodes. The gels were stained with 1% amido black in 7% acetic acid for 1 hour and then destained overnight in 7% acetic acid. The destained gels were then scanned at 550 nm on a Beckman DU Spectrophotometer for protein bands and the results were recorded on a Gilford 2000 Recorder.

Absorption spectrum of cytochrome c-551

Absorption spectra of cytochrome c-551 were taken in the following manner on a Cary 17 D Spectrophotometer.

1. The sample was diluted appropriately with .01 M phosphate buffer, pH 7.0 and adjusted to pH 7.0 if necessary.

2. A baseline of buffer versus buffer was recorded from 600 nm to 400 nm.

3. The sample was oxidized by the addition of 50 microliters of fresh 1 mM $K_3Fe(CN)_6$ and a spectrum was recorded from 600 to 400 nm.

4. The sample was reduced with a few grains of sodium dithionite and the spectrum was recorded as before.

Determination of enzymatic activity of cytochrome c-551

A dilute solution of cytochrome oxidase from *Pseudomonas aeruginosa* P6009 was made by adding .25 grams of the highly purified cytochrome oxidase to 5 ml of 0.01 M phosphate buffer, pH 7.0. This solution was then oxidized by the addition of a few crystals of $K_3Fe(CN)_6$. 2.5 ml were then pipetted into a reaction vessel connected to a Clark oxygen electrode (Yellow Springs Instrument Co.). The oxygen uptake was recorded for six minutes. A few grains of solid ascorbic acid (Sigma) were then added to the reaction vessel. After six minutes, 0.5 ml of a highly purified cytochrome c-551 solution from the experimental organism was added to the reaction vessel and the oxygen uptake was recorded.

Electron microscope technique of whole mounted bacteria

A 1:1 mixture of a bacterial suspension and 2% phosphotungstic acid (pH 6.8) was prepared for the purpose of negative staining (Kay, 1967). A small drop of the mixture was placed on collodion coated, copper grids and observed with a Hitachi HS-8 transmission

electron microscope.

Electron microscope technique of sectioned bacteria

Bacterial cells were fixed in Kellenberger's standard fixative (Kay, 1967), dehydrated in ethanol and embedded in Araldite 6005. Sections approximately 600 angstroms thick were double stained with lead and uranium salts and observed under the transmission electron microscope.

RESULTS AND DISCUSSION

Studies with a transmission electron microscope indicated the bacterium used for the present study appeared to be a gram-negative, lophotrichously flagellated bacillus. The Gram test confirmed the Gram-negative character of the experimental organism (Figs. 1 & 2, Appendix A).

When bacterial cells were collected, it was noticed that the harvested cells appeared reddish in color. On the basis of this color, it was suspected that the reddish tinge was due to the presence of cytochromes. A crude cell free extract was made by sonification of the harvested cells and a spectrum of the reduced extract was taken. The extract contained absorption maxima at 551 nm, 522 nm, and 418 nm. This was compared to literature values of 550 nm, 521 nm, and 415 nm for the well-studied horse heart cytochrome c (Dickerson and Timkovich, 1975). Purification and characterization of this c-type cytochrome ensued. The initial spectrum was extremely noisy because of low content of cytochromes as well as the presence of many other protein contaminants. However, purification of the cytochrome c-551 from the experimental organism was certainly expected to improve the absorption spectrum of the enzyme.

In order to purify c-551, it was necessary to grow large amounts of the bacteria. A nutritional study was conducted to find a medium in which the organism would grow well and produce a large amount of cytochromes. One liter each of six different media were prepared and innoculated and aerobically grown at 25° C for 24 hours on a gyrotory shaker. The same six media were similarly prepared and grown for 3 days

at 25° C under anaerobic conditions. The 12 flasks were harvested and the respective dry weights of the bacterial cells were determined (Table 1, Appendix A). The results show that the anaerobically grown cells showed little if any growth. Certain species such as Pseudomonas aeruginosa, a denitryfying bacterium, produces large quantities of cytochrome c under anaerobic conditions in the presence of nitrate as a terminal electron acceptor (Horio et al., 1960). The experimental organism was obviously different in this respect. The phthalate medium was found to produce only minimal quantities of cells. The top three media for growth of the test bacterium were Citrate-Nitrate, Phthalate-Glutamate, and Nutrient Broth.

The aerobically grown cells from six different media were collected into 20 ml and difference spectra were recorded. Visual examination of the height of the alpha peak at 551 nm readily showed that Nutrient Broth and the Citrate-Nitrate media produced the largest quantities of cytochrome c-551.

These two media were compared on two different dates for the production of cytochromes. The results of these studies are shown in Table 2 (Appendix A). The method of Kodama and Shidara (1969) was used to estimate cytochromes c-551 and b-558 in the whole cells. It was found on both dates that the Nutrient Broth medium produced approximately twice as much cytochrome c-551 as did the Citrate-Nitrate medium in terms of micromoles of c-551 per gram dry weight of bacteria. It is interesting to note that the Citrate-Nitrate medium produced nearly twice as much cytochrome b-558 on both dates. It was obvious that nutrition influences the relative concentrations of the various cytochrome components of this

bacterium's electron transport chain. Nutrient broth was selected as the media which would produce the largest quantity of cytochrome c-551 for future purification studies.

The biggest problem in purification of cytochrome c from bacteria has been the breakage of the cell wall and extraction of cytochrome c from the membrane. Once the protein is in solution, purification is relatively easy. A variety of techniques were performed in earlier extraction experiments. Sonification of the cells was not considered a good procedure for the experimental organism. It was marred by low yields of cytochrome c and the production of small membrane-bound vesicles containing cytochrome c which could not be released easily. Ambler (1963) used alumina and blending for preparation of cell free extracts from *Pseudomonas aeruginosa*. This method resulted in low yields of c-551 and protein concentrations of around 5 mg/ml with the experimental organism. The method chosen for the final purification procedure was disruption of the cell wall by a French pressure cell. This resulted in cell free extracts that contain nearly 90 mg/ml of protein.

Ammonium sulfate fractionation of cell free extracts showed that the 30-80% fraction contained all of the measurable cytochrome c-551. This method resulted in slight purification of cytochrome c-551, and was the second step of the purification procedure.

The fraction from the ammonium sulfate step was subjected to the third step in the purification procedure, namely SP-Sephadex batch-wise chromatography. SP-Sephadex was chosen as an ion-exchanger because of its negative charge over a wide range of pH from 2.0 to 10.0. This would

be an ideal ion-exchanger for a batch procedure at low pH where the cytochrome has a net positive charge and would bind to the resin. As the pH is increased past the isoelectric point of the enzyme, the bound cytochrome becomes repelled as it becomes more negative and is expected to be eluted from the column. After many trials, it was found that cytochrome c-551 was bound firmly to the resin at pH 3.9 in 0.01 M phosphate buffer and eluted readily by increasing the pH to 10.0.

Gradient chromatography was attempted after the batch techniques. An early experiment with DEAE-cellulose indicated the possible existence of two different types of cytochrome c-551 (Fig. 3, Appendix A).

PH gradients were tried early in the research, but were not attempted further as only limited success was obtained.

Gradient chromatography with SP-Sephadex was chosen as the fourth step in the purification procedure. The linear gradient between the starting buffer and the same buffer containing 2.0 M NaCl eluted the cytochrome in a broad, diffuse band. This was a poor step in improving the purity of the c-551 with considerable loss of the protein. Gel electrophoresis was performed at this stage. The results indicate the presence of at least 7 proteins (Fig. 4, Appendix A). The degree of purity at this stage is not extremely high, approximately 10 times the original cell free extract, as can be seen in Table 4 (Appendix A). It is felt that possibly cytochrome c-551 is not even represented by any of the bands on the gels. There were no visable orange bands on the gels and cytochrome c-551 is, at best, a very minor component of the protein mixture at this step.

Polyacrylamide gel isoelectric focusing was performed and the

isoelectric point of the cytochrome c-551 was determined (Fig. 6, Appendix A). Cytochrome c-551 was seen on sample gels at 6.0 cm from the top of the gel. Absorbance scans of the gels at 410 and 551 nm showed that the absorption peaks at 6.0 cm of the gel were due to cytochrome c-551. The plotted pH gradient indicated a pH of 6.1 at this region of the gel. This was the same pH recorded after cutting out the brown cytochrome band in another gel after the ampholytes had diffused out from the gel. A spectrum of this solution showed absorption maxima identical to the experimental organism's c-551. All results indicate that the isoelectric point of cytochrome c-551 is 6.1. This knowledge was found to be extremely useful in the next procedure, preparative isoelectric focusing.

Preparative isoelectric focusing was performed with a wide section of the column made from pH 5.0 to 7.0 to take advantage of the knowledge of the isoelectric point of cytochrome of c-551. A sharp band corresponding to the oxidized and reduced forms of the enzyme was collected.

The purification procedure for cytochrome c-551 is shown in Table 4 (Appendix A). From 60 grams of lyophilized bacteria, nearly 40 grams of protein was found in the cell free extract. This indicates an efficient extraction procedure. It can be seen that the major purification occurred with preparative isoelectric focusing. The degree of purification of the enzyme is nearly 250 times the original cell free extract. The highly purified sample was analyzed for purity on gel electrophoresis (Fig. 5, Appendix A). The main peak belongs to cytochrome c-551 whose position on the gel could easily be seen during the course of the electrophoresis on the unstained gel and the observed position concurs with peak number 3.

It is likely that many of the other peaks represent cytochrome c-551 complexed with the carrier ampholytes which appeared to be not completely dialyzed out. Pure compounds have been known to show 5 different peaks after similar experimental conditions (Richard Marks, personal communication). It was estimated that cytochrome c-551 is probably 50% pure, assuming a molecular weight of 10,000. Other fractions from the isoelectric focusing experiment seemed to confirm the interaction of carrier ampholytes and cytochrome c-551 as minute quantities of c-551 were found in several fractions with widely differing pH values. It is unclear whether the possible existence of two forms of cytochrome c-551 as indicated from the DEAE-cellulose experiment (Fig. 3, Appendix A) is a valid assumption.

The spectrum of a highly purified sample of cytochrome c-551 from the experimental organism can be seen in Fig. 7 (Appendix A). There exists an oxidized Soret band at 408 nm. The reduced spectrum possesses a 551 nm alpha peak, a beta reduced maxima at 522 nm, and a 416 absorbance maxima at the reduced Soret band. This is the classical absorption spectrum that characterizes the enzyme. It is a major improvement over the "noisy" spectra recorded at the start of the research. From comparison with other c-type cytochromes, the cytochrome c-551 of the experimental organism seems to belong to the broad class of c-type cytochromes similar to other prokaryotic and eukaryotic cytochrome c (Table 3, Appendix A).

An enzymatic experiment with a highly purified sample of cytochrome c-551 was carried out to see if the enzyme that was being isolated was still functional as an electron carrier. To a solution of ascorbate, cytochrome oxidase from _Pseudomonas aeruginosa_, the c-551 from the experimental

organism was added and oxygen uptake was measured with an oxygen electrode. As can be seen in Fig. 8 (Appendix A), electron transport from ascorbate to oxygen via cytochrome oxidase was not observed unless cytochrome c-551 was added. The results indicate that the cytochrome c-551 used in this study was enzymatically functional. Results confirm also the conservative aspect of evolution in which a mode of interaction between cytochrome c and cytochrome oxidase is maintained, for cytochrome c and cytochrome oxidase from different organisms reacted readily. They are furthermore an evidence for the experimental organism being closely related to *Pseudomonas aeruginosa*.

SUMMARY

Routine inspection of the harvested bacterial cells from a phthalate metabolizing bacterium showed a reddish tint which was thought to be due to the presence of cytochromes. This report describes purification and characterization of cytochrome c from this organism.

Nutrient broth was chosen as the growth medium since it produced the largest quantities of cytochrome c-551 per gram dry weight of bacteria. It was found also that nutrition plays a role in the relative concentrations of cytochrome c-551 and cytochrome b-558 in the experimental organism.

The French Press was found to be very efficient in liberating the cytochrome c-551 from the bacterial cells. The extracted c-551 was further purified with ammonium sulfate fractionation and ion-exchange techniques with SP-Sephadex. The major purification resulted from isoelectric focusing which resulted in 250 fold purification over the original cell free extract. Gel electrophoresis confirmed the purity of the final product was very high.

The cytochrome c-551 had reduced maxima at 551, 522, and 416 nm. The oxidized peak was at 408 nm. The protein appeared to be a typical cytochrome c similar to many of the prokaryotic c-type cytochromes and mitochondrial cytochrome c.

Evidence of electron transport capabilities of the highly purified enzyme indicated that the isolation procedures had not damaged the enzymatic functions of the cytochrome c-551.

APPENDIX

APPENDIX A

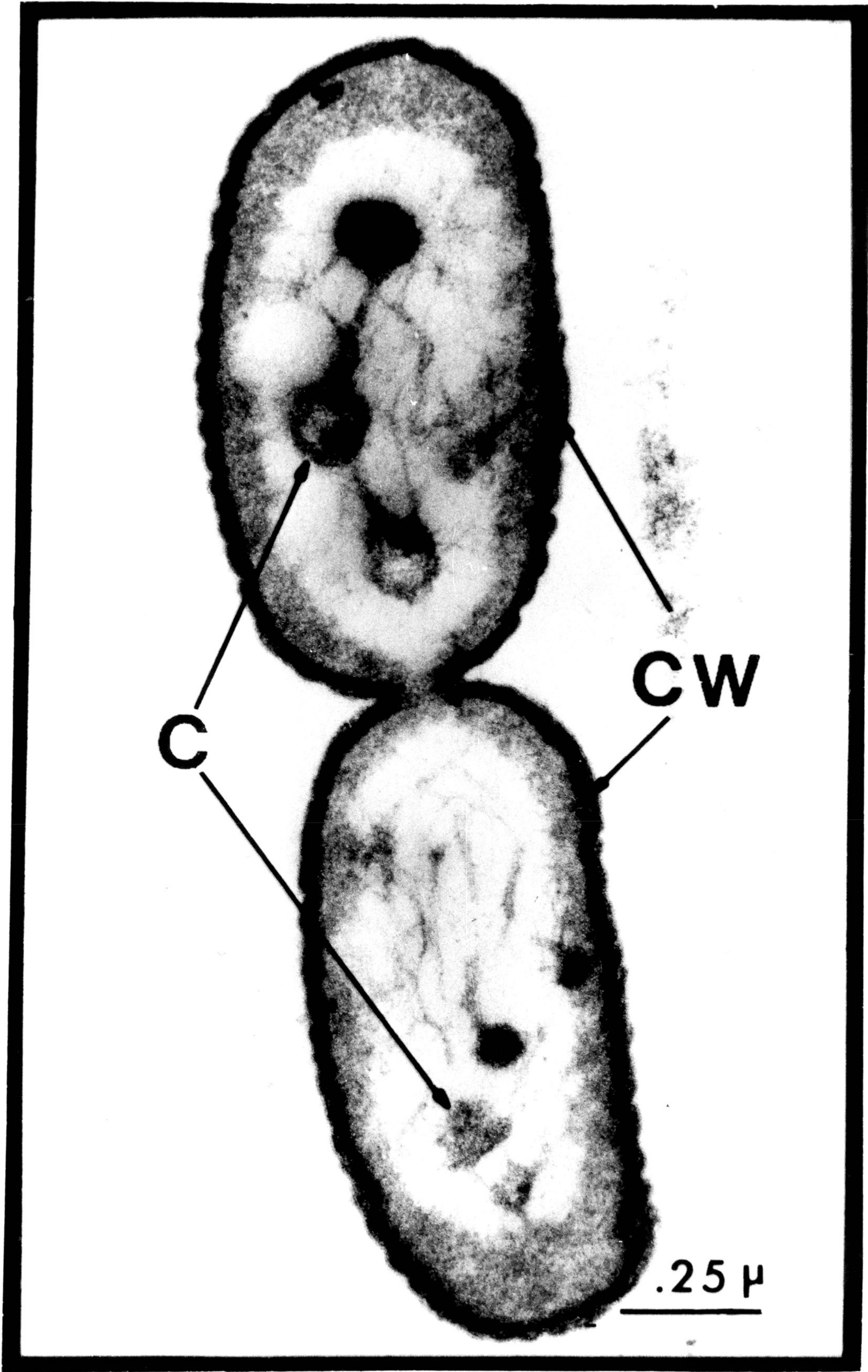
Fig. 1. Electron micrograph of a sectioned phthalate metabolizing bacterium undergoing binary fission X 96,000.

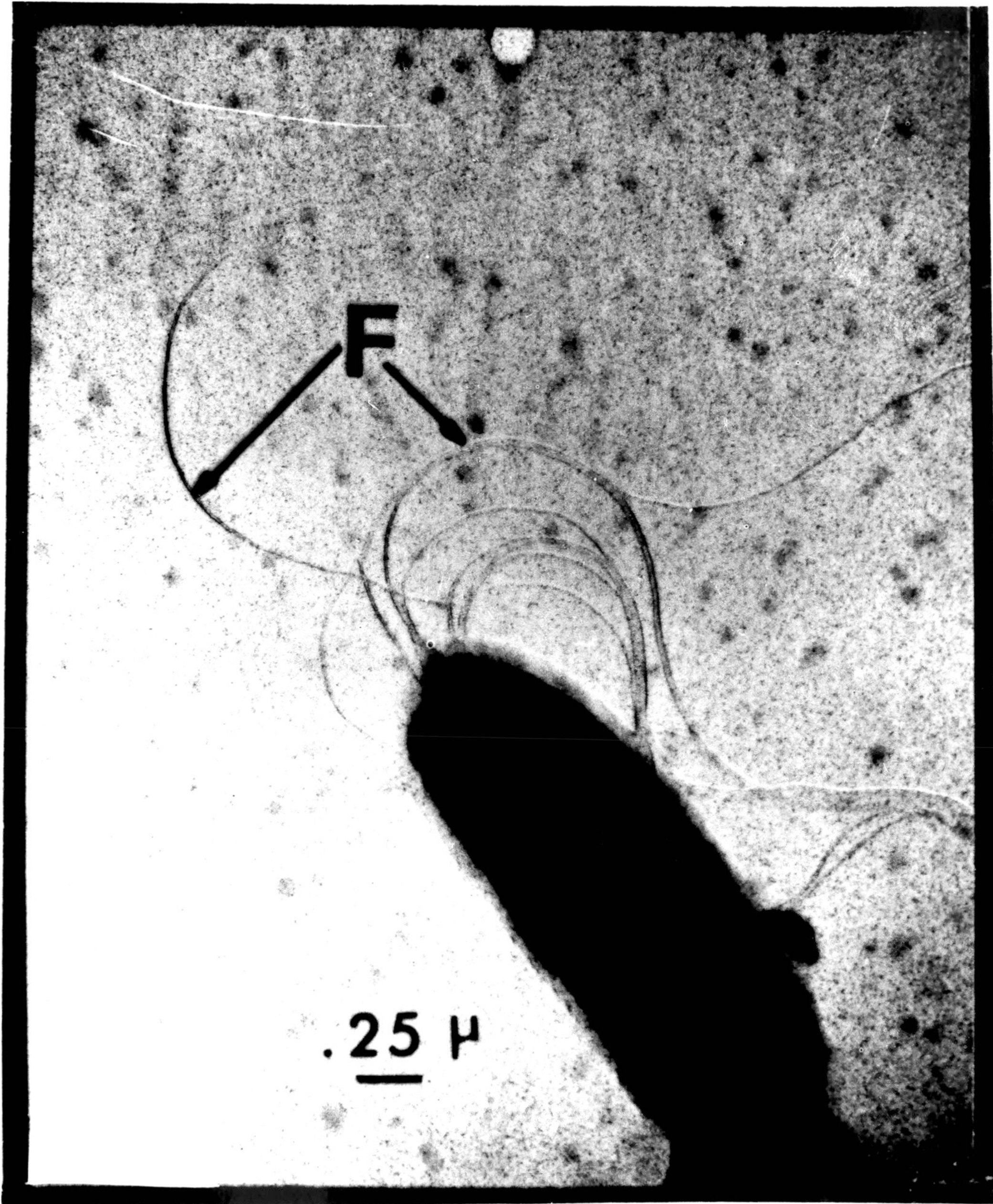
Fig. 2. Negatively stained whole mount preparation of the phthalate bacterium X 45,000.

F = flagellum

CW = cell wall

C = chromatin


CW
C
.25 μ


F
.25 μ

APPENDIX A (Continued)

Table 1. Results of the dry weight of bacteria produced per liter of medium under aerobic and anaerobic conditions using different media. NG*, no growth.

APPENDIX A (Continued)

TABLE 1

NUTRITIONAL STUDY

MEDIUM	AEROBIC DRY WEIGHT (GRAMS/LITER)	ANAEROBIC DRY WEIGHT (GRAMS/LITER)
PHTHALATE	0.124	NG*
PHTHALATE-GLUTAMATE	0.668	NG
PHTHALATE-GLUTAMATE-NITRATE	0.467	NG
NUTRIENT BROTH	0.576	0.062
NUTRIENT BROTH-NITRATE	0.485	0.131
CITRATE-NITRATE	0.754	0.111

APPENDIX A (Continued)

Table 2. Results of a comparison of Nutrient Broth and Citrate-Nitrate media for production of cytochrome c-551 and cytochrome b-558 with the experimental organism on two different dates. The results are expressed in terms of micromoles of cytochrome produced per gram dry weight of bacteria.

APPENDIX A (Continued)

TABLE 2

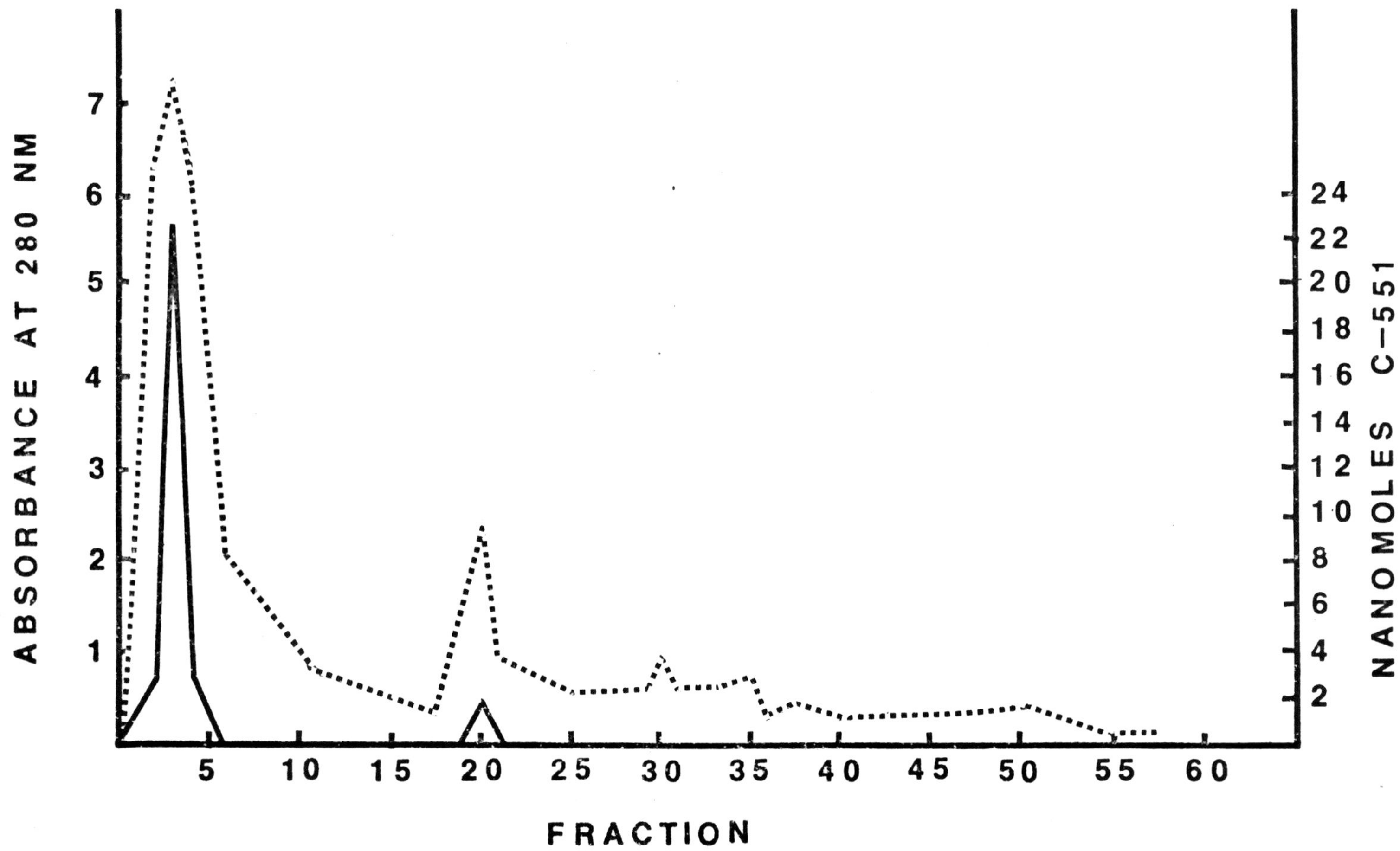
COMPARISON OF TWO MEDIA FOR CYTOCHROME PRODUCTION

MEDIUM	MICROMOLES CYTOCHROME C-551 PER GRAM DRY WEIGHT OF BACTERIA*	MICROMOLES OF CYTOCHROME B-558 PER GRAM DRY WEIGHT OF BACTERIA*
NUTRIENT BROTH		
10/25/77	0.354	0.151
10/31/77	0.475	0.231
CITRATE-NITRATE		
10/25/77	0.083	0.255
10/31/77	0.240	0.355

*Calculated by the method of Kodama and Shidara (1969).

APPENDIX A (Continued)

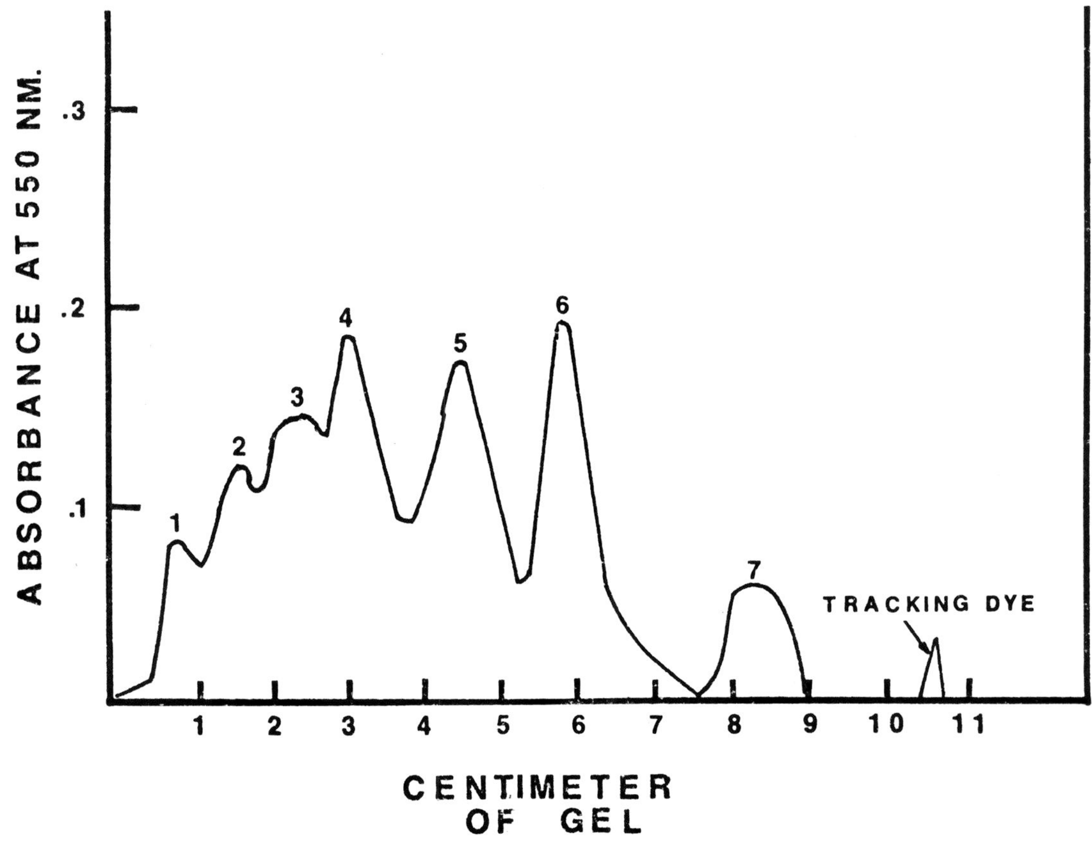
Fig. 3. Results of an ion-exchange experiment using DEAE-cellulose. The initial concentration of the eluting buffer was .05 M phosphate buffer, pH 7.4. The salt concentration was steadily increased by the continual mixing of the starting buffer with a 2.0 M NaCl in the same buffer. The abcissa indicates 3.0 ml fractions eluted during the course of the experiment. The fractions were analyzed spectrophotometrically for absorbance at 280 nm which indicates protein, and assayed for cytochrome c-551. This is indicated by the left and right ordinate respectively. The results indicate the possible existance of two forms of c-551. The solid line, 551 nm. The dotted line, 280 nm.

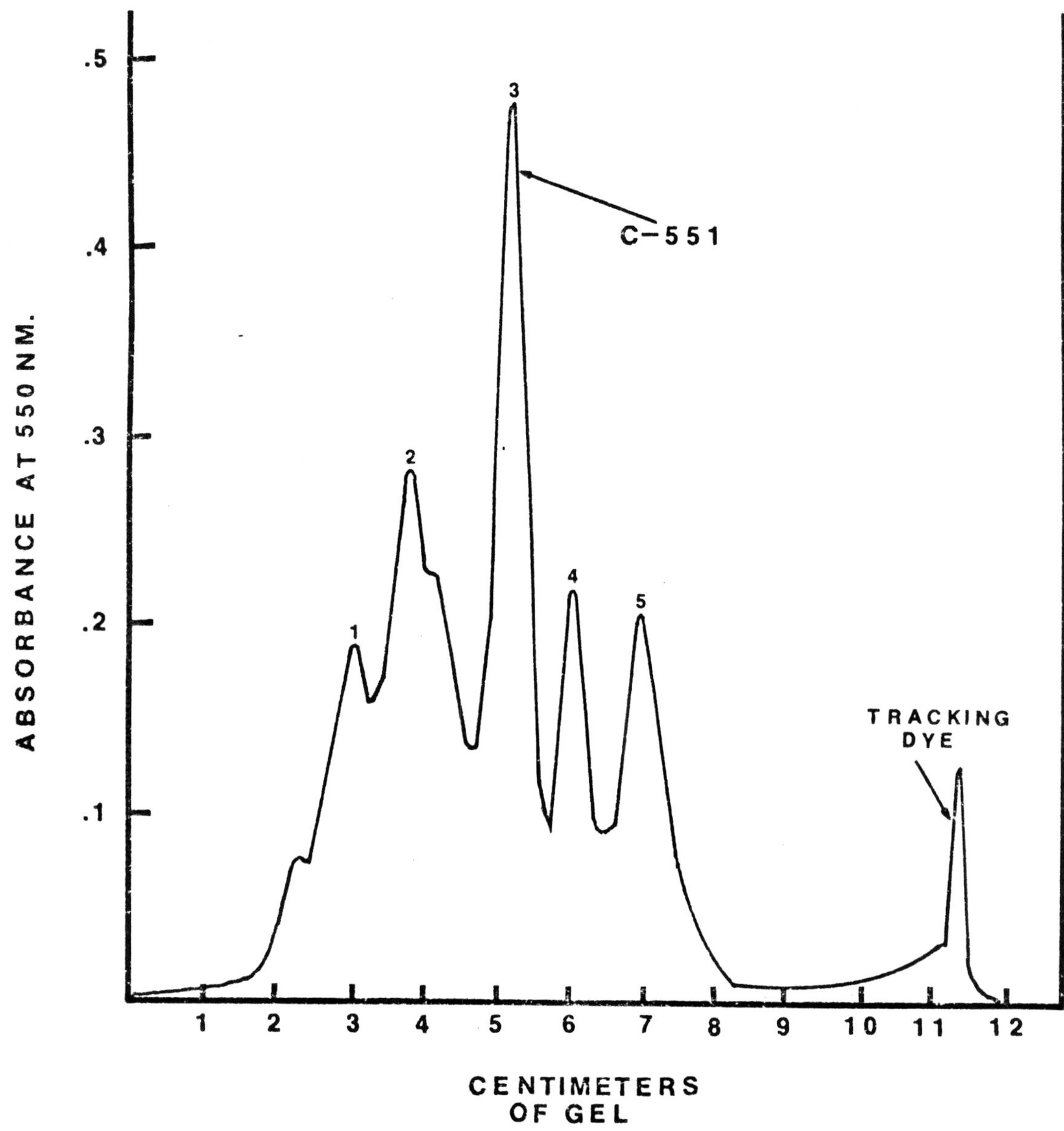

ABSORBANCE AT 280 NM
1
2
3
4
5
6
7
NANOMOLES C-551
2
4
6
8
10
12
14
16
18
20
22
24
5
10
15
20
25
30
35
40
45
50
55
60
FRACTION

APPENDIX A (Continued)

Fig. 4. Results of gel electrophoresis on the cytochrome c-551 fraction from the SP-Sephadex gradient chromatography to determine the relative purity. The results indicate the presence of at least 7 compounds. None of the peaks could be attributed to cytochrome c-551. It was felt that cytochrome c-551 was at best a minor component of the protein mixture at this point.

Fig. 5. Results of gel electrophoresis on the cytochrome c-551 fraction from the preparative isoelectric focusing experiment. A large, distinct band that was obviously cytochrome, could be visually seen on the unstained gels at 5.0 cm from the top of the gel. This corresponds to peak 3 which is shown as the major component. The other peaks might possibly represent complexes with c-551 with the carrier ampholytes along with minor proteins contaminants. It is felt that the cytochrome c-551 is at least 50% pure at this stage. The peaks that could be visually seen on the stained gels are numbered as before.

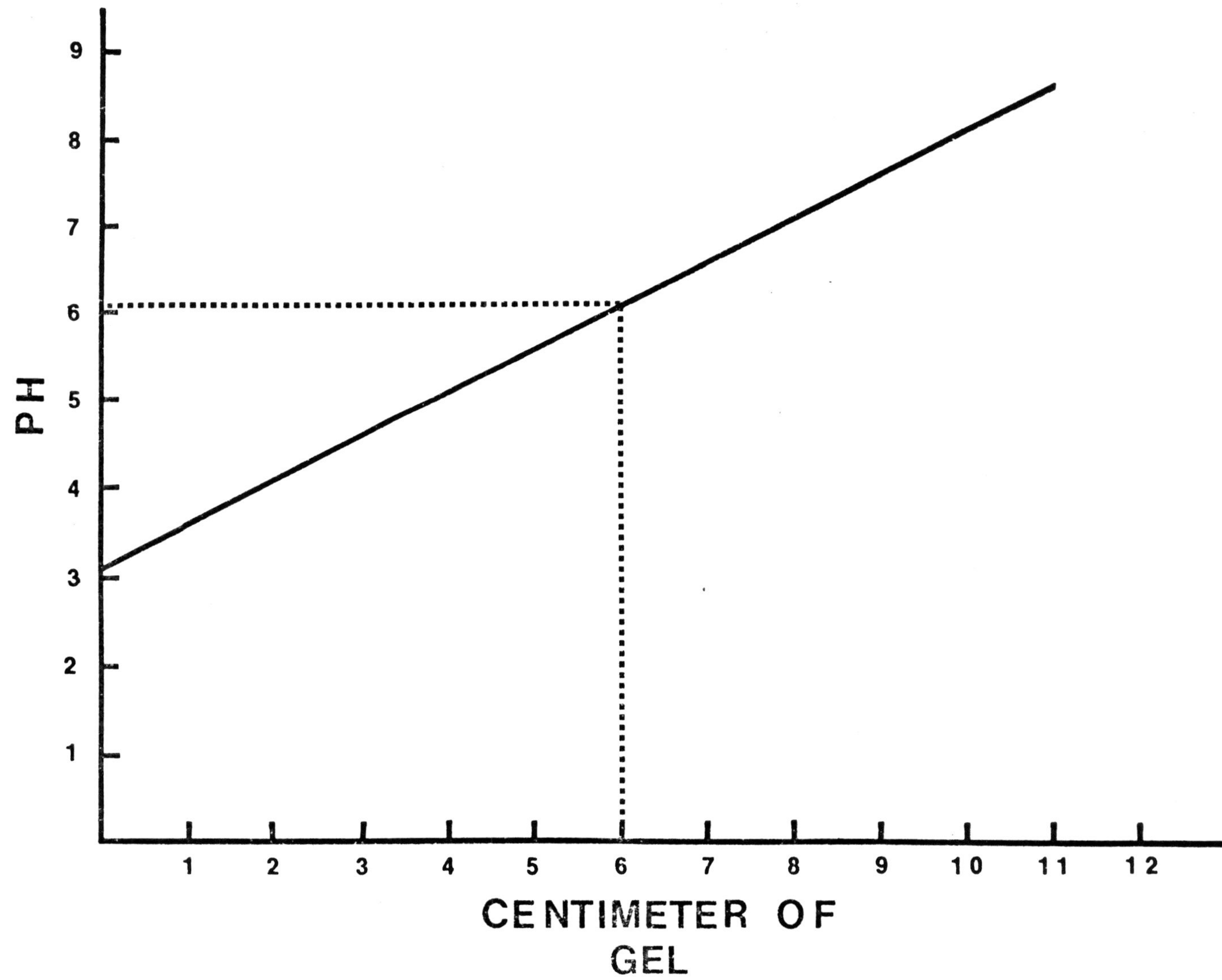

ABSORBANCE AT 550 NM.
.3
.2
.1
1
2
3
4
5
6
7
TRACKING DYE
1
2
3
4
5
6
7
8
9
10
11
CENTIMETER
OF GEL



ABSORBANCE AT 550 NM.
.5
.4
.3
.2
.1
1
2
3
4
5
C-551
TRACKING DYE
1 2 3 4 5 6 7 8 9 10 11 12
CENTIMETERS OF GEL

APPENDIX A (Continued)

Fig. 6. The graph of the pH gradient over the length of the gel is shown for the polyacrylamide gel electrofocusing experiment. Cytochrome c-551 was detected spectrally by scanning the gels at 410 and 551 where cytochrome c-551 absorbs heavily. It was found to be 6 cm from the top of the gels. This corresponds to a pH of 6.1 for that region of the gel. This is the isoelectric point of the enzyme. This was also confirmed by cutting out the visual brownish cytochrome band and taking the pH of the gel after letting it stand in deionized water for 24 hours. The solution was assayed for cytochrome c-551 and was found to contain absorption peaks which are identical to cytochrome c-551 of the experimental organism.



9
8
7
6
5
4
3
2
1
PH
1
2
3
4
5
6
7
8
9
10
11
12
CENTIMETER OF
GEL

APPENDIX A (Continued)

Table 3. Comparison of various c-type cytochromes from selected organisms are shown. It is obvious that the experimental organims possesses a c-type spectrum that is extremely similar to other cytochromes c. It is speculated that the experimental organism is evolutionarily related to a broad group of cytochrome c which includes horse heart and *Pseudomonas aeruginosa* cytochrome c.

APPENDIX A (Continued)

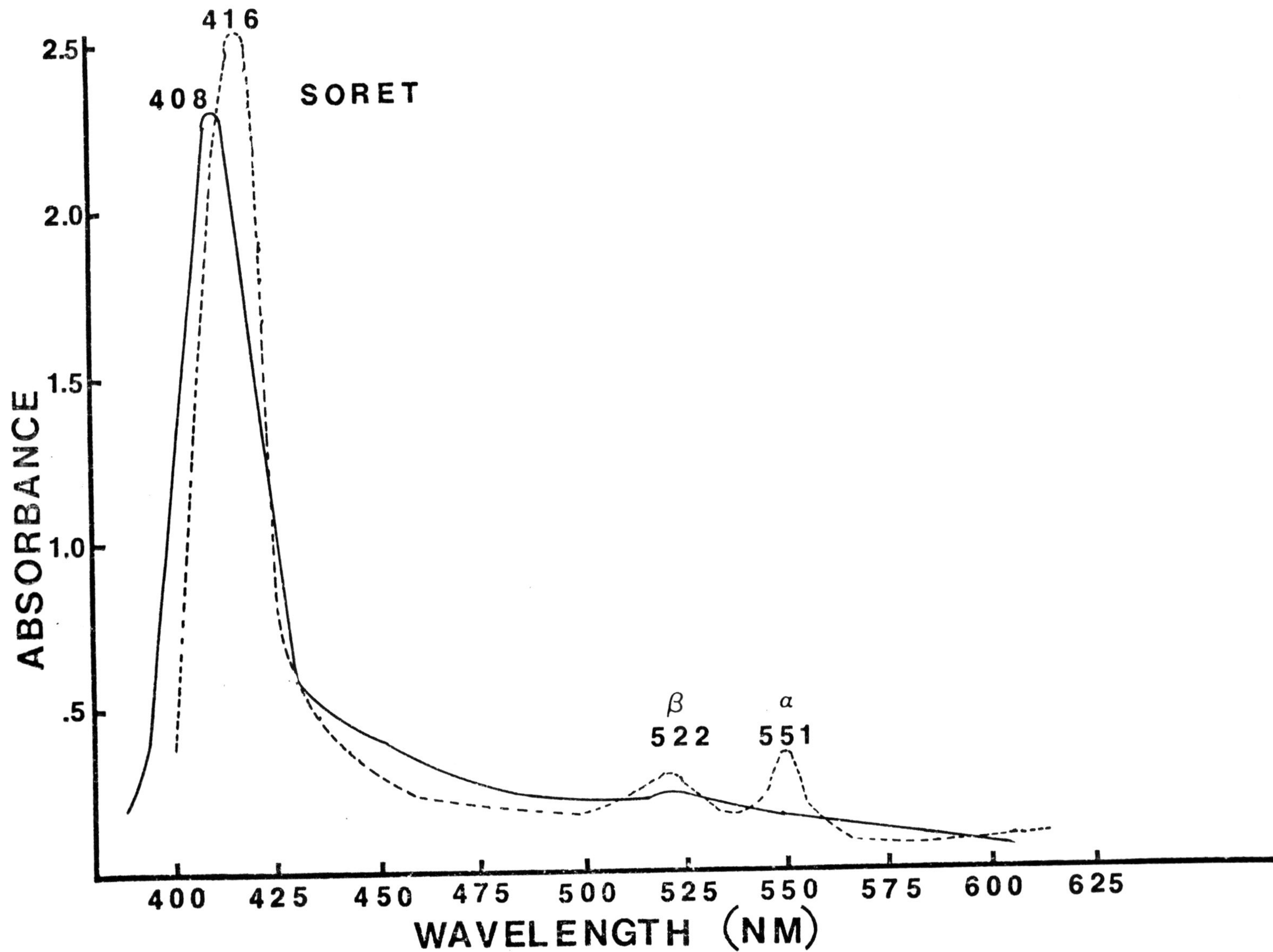
TABLE 3

COMPARISON OF C-TYPE CYTOCHROMES

TYPE	SOURCE	ABSORPTION MAXIMA (REDUCED)	ABSORPTION MAXIMA (OXIDIZED)	ISOELECTRIC POINT
C_2	*Rhodospirillium rubrum*	550,521,415	410	6.2
Flavin	*Chromatium* (Strain D)	552,523,416	410	4.5
C-551	*Pseudomonas aeruginosa*	551,521,416	409	4.7
C	Horse heart	550,521,415	410	10.0
C_6	Green plant photosynthetic	554,524,422	-	-
C-551	Experimental Organism	551,522,416	408	6.1

APPENDIX A (Continued)

Fig. 7. Oxidized and reduced spectra of a highly purified cytochrome c-551 sample. The solid line represents the oxidized absorption spectrum of the enzyme. It exhibits an absorbance peak at 408 nm. The reduced spectrum is shown by the dotted line. The cytochrome c-551 has absorption maxima at 551, 522, and 416 nm in the reduced state. This is a classical c-type spectrum.


416
408
SORET
2.5
2.0
1.5
1.0
.5
ABSORBANCE
β
522
α
551
400
425
450
475
500
525
550
575
600
625
WAVELENGTH (NM)

APPENDIX A (Continued)

Table 4. Summary of purification of cytochrome c-551.

APPENDIX A (Continued)

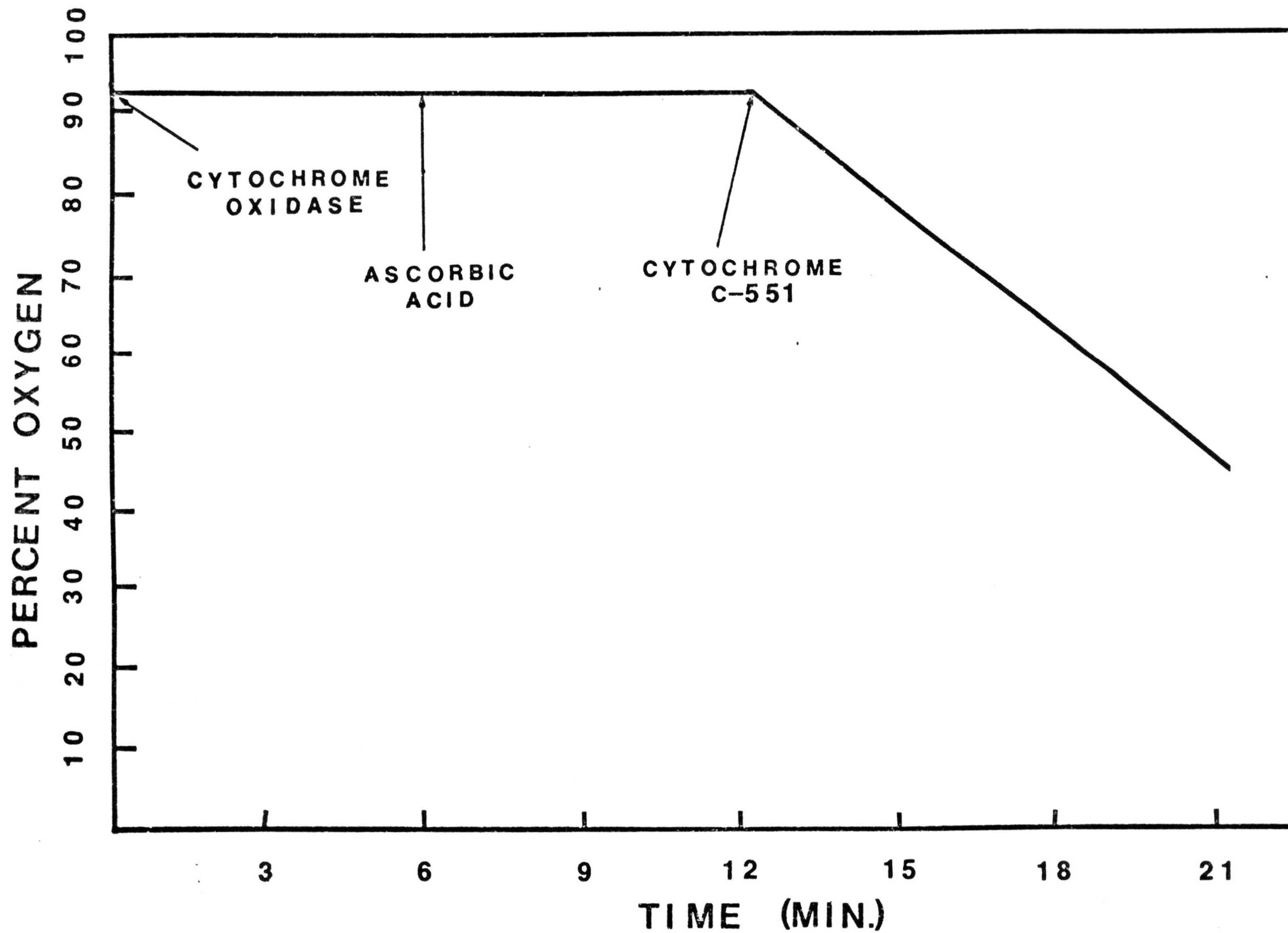
TABLE 4

PURIFICATION STEPS

STEP	VOLUME (ml)	NANOMOLES OF CYTOCHROME C-551	PROTEIN (mg)	NANOMOLES PER MG PROTEIN	FOLD PURIFICATION
CELL FREE EXTRACT	435	7,010	39,142	.179	-
30-80% AMMONIUM SULFATE FRACTION	390	4,450	19,449	.229	1.28
BATCH EXTRACTION WITH SP-SEPHADEX	416	956	667	1.430	7.99
GRADIENT CHROMATOG-RAPHY WITH SP-SEPHADEX	200	250	137	1.825	10.20
PREPARATIVE ISO-ELECTRIC FOCUSING	6.5	162	3.66	44.26	247.3

APPENDIX A (Continued)

Fig. 8. Results from the enzymatic experiment are shown. The graph shows the oxygen uptake after cytochrome c-551 was added to a reaction vessel connected to an oxygen electrode which showed no oxygen uptake after cytochrome oxidase from *Pseudomonas aeruginosa* and ascorbate had been added to the reaction vessel. The results indicate that the isolated cytochrome c-551 is still a functional enzyme possessing electron transport capabilities.


PERCENT OXYGEN
100
90
80
70
60
50
40
30
20
10
CYTOCHROME
OXIDASE
ASCORBIC
ACID
CYTOCHROME
C-551
3
6
9
12
15
18
21
TIME (MIN.)

APPENDIX B

Table 1. List of Abbreviations.

APPENDIX B (Continued)

Table 1.

List of Abbreviations

Bis	N, N'-methylenebisacrylamide
C.	Centigrade
cm	centimeter
DEAE-cellulose	diethylaminoethylcellulose
g	gram or gravity
M	Molar
mg	milligram
ml	milliliter
mM	millimolar
mm	millimeter
N	Normality
nm	nanometer
SP-Sephadex	Sulfopropyl-Sephadex
Temed	N, N, N,' N'-tetramethylethylenediamine

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