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THE CYTOCHROME COMPOSITION OF
BACILLUS SUBTILIS AND BACILLUS MEGATERIUM

A THESIS
SUBMITTED TO THE
DEPARTMENT OF CHEMISTRY
BRIGHAM YOUNG UNIVERSITY
PROVO, UTAH

IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

BY
JOHN H. MANGUM
AUGUST, 1959

This Thesis by John H. Mangum is accepted in its present form by the Department of Chemistry of the Brigham Young University as satisfying the thesis requirements for the degree of Master of Science.

July, 1959
Date

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This thesis is dedicated by the author to his wife, Sandra, whose encouragement during this investigation was and always will be appreciated.

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INTRODUCTION

Of all the heme pigments found in nature, the cytochromes are perhaps the most widely distributed (1). Their presence has been demonstrated in various types of cells of animals and higher plants, and in most bacteria. Many of the early investigations were carried out with baker's yeast and with preparations of heart muscle tissue (2). Cytochrome c from both these sources has been extensively purified (3). The present knowledge concerning the bacterial cytochromes has been derived primarily from spectroscopic observations of either whole cell suspensions or crude extracts. It was not until 1953 that the first report on the isolation of a bacterial cytochrome appeared (4). Since then a number of bacterial cytochromes have been obtained in highly purified form.

Several investigations have demonstrated that the cytochrome composition of Bacillus subtilis and Bacillus megaterium was very similar to that of yeast and mammalian tissue (5,6). The alpha bands of the reduced pigments were at 605, 564, and 551 mμ., corresponding to a mixture of cytochromes $a + a_3$, b, and c, respectively. The beta bands overlapped to form one inhomogeneous band around 525 mμ. The gamma bands (also called the Soret bands) were at 445 mμ., (cytochromes $a + a_3$), 430 mμ. (cytochrome b) and at 415 (cytochrome c) in the reduced form. Because of this similarity to mammalian tissue and yeast an investigation of these bacterial cytochromes was undertaken, in the hope that the various cytochrome components could be purified and characterized. If this could be accomplished, it would be possible to compare the properties of these bacterial cytochromes with those obtained from yeast or mammalian tissue.

Since current research now clearly shows that the respiratory enzymes are responsible for the most important energy transformations of the cell, it was decided to study several of these enzymatic processes involving the cytochromes. DPNH oxidase, succinic dehydrogenase, cytochrome c reductase, and cytochrome oxidase activities have been demonstrated in many

crude and a few partially purified bacterial extracts. Ruptured cells of *B. megaterium* were assayed for these enzymes.

LITERATURE REVIEW

In 1885, Mac Munn (7) observed spectroscopically that many tissues contained pigments with absorption characteristics similar to hematin (ferriprotoporphyrin) and its derivitives and named them histohematins. In 1925, Keilin (1) published his first paper on these pigments. He also noted their widespread distribution in nature. Keilin renamed these pigments the cytochromes (cell pigments) to avoid the earlier confusion of nomenclature.

In the years which have elapsed since Keilin's first work on the cytochromes, an extensive investigation of these pigments has been made by a multitude of investigators. Many of the early studies were made with baker's yeast and with a preparation of heart muscle extract devised by Keilin and Hartree (2). The cytochrome systems of yeast and heart muscle were found to be similar. The alpha bands of the reduced pigments were seen at 605, 564, and 551 mμ., corresponding to a mixture of cytochromes $\underline{a} + \underline{a}_3$, $\underline{b}$ and $\underline{c}$ respectively. These cytochromes also showed secondary or beta bands which overlap to form one unsymmetrical band around 525 mμ. The gamma bands were located at 445 mμ. (cytochromes $\underline{a}+\underline{a}_3$), 430 mμ. (cytochrome $\underline{b}$), 415 mμ. (cytochrome $\underline{c}$) when these pigments were in the reduced form. Keilin and Hartree (8) observed that cytochrome $\underline{a}_3$ had a number of properties relating it to the enzyme cytochrome oxidase, and that the absorption bands of its carbon monoxide compound corresponded to those observed by Kubowitz and Haas (9) for their "respiratory enzyme". Chance (10) definitely showed that cytochrome $\underline{a}_3$ of baker's yeast and heart muscle was the cytochrome oxidase of these cells.

Theorell (3) was the first to isolate and purify cytochrome $\underline{c}$. Since that time, cytochrome $\underline{c}$ has been purified from a number of sources; thus its properties have been extensively studied. The other cytochromes have not been as completely investigated. Very little success has attended those investigators who have attempted to isolate and characterize $\underline{b}$ type cytochromes. Horio *et al.* (52-54) have purified a water soluble cytochrome oxidase from *Pseudomonas aeruginosa*. However, its specificity to other cytochromes was different from that of animal cytochrome $\underline{c}$ oxidase.

It could oxidize animal and yeast ferrocytochrome of type c only slowly. Connelly et al. (11) succeeded in obtaining a preparation of cytochrome oxidase from pig heart which was suitable for electrophoretic studies. Column electrophoresis of the preparation showed that at least partial separation of the hemoprotein having cytochrome oxidase activity from inactive hemoprotein had occurred. From these and many similar studies has come a knowledge of the function of cytochrome oxidase. It acts as a terminal oxidase in linking the electron transport chain to molecular oxygen. A review of the mammalian cytochrome system has been written by Keilin and Slater (12). Wyman's review of the hemoproteins (13) is concerned with the structure and properties of these pigments. An excellent review article dealing with bacterial cytochromes was written in 1954 by Lucile Smith (14). She has attempted to compare and contrast the bacterial cytochromes with those of mammalian tissues.

The bacterial cytochromes have not been investigated as thoroughly as have the mammalian cytochromes. Early experimenters (15, 16) reported that bands of the reduced cytochromes were not at the same wavelengths as those of yeast and mammalian tissues. Some bacteria were reported to contain no cytochromes, and this led to the suggestion that a relationship existed between the cytochrome composition and the degree of aerobiosis. However, this has been disproven by subsequent investigation (53).

There was considerable disagreement among the many spectroscopic examinations of the cytochromes of bacteria. However, the observations of several investigators (17-28) can be summed up in the following manner:

(a) Bacillus subtilis, Bacillus pertussis, Sarcina lutea, Bacillus pycocyaneus and some other mycobacteria have absorption spectra which resemble those of yeast or mammalian tissues.

(b) In Escherichia coli, Azotobacter chroococcum, Acetobacter pasterianum, and Acetobacter peroxydans, cytochrome a was often found to be missing and was replaced by cytochrome a_1 and a_2.

(c) In E. coli and Proteus vulgaris the combination of cytochrome b and c seemed to be replaced by cytochrome b_1.

(d) A number of species of streptococci and pneumococci and obligate anerobes were reported to contain no cytochromes. However, cytochromes have been found in Chlorobium limicola and Desulfovibrio desulfuricans.

While this list is by no means exhaustive, its purpose is to indicate the diverse nature of the cytochrome complements of bacteria.

The disagreement aluded to earlier for different spectroscopic examinations of the bacterial cytochromes may well be due to variations among the different strains of bacteria or to different culturing conditions. Smith has reviewed (14) the observations made concerning these variations of bacterial cytochromes. In addition to the articles reviewed by Smith, Gary (27) reported that there is some difference in the cytochrome oxidase activity of B. subtilis depending upon the type of media chosen to grow the cells. Chaix and Flamens (28) observed that cultures of Bacillus coagulans grown under completely anaerobic conditions did not contain cytochromes, while aerobically grown cultures contained cytochromes a and b. Cytochrome c was not present in these cultures even after growth for four to five hours. Semi-aerobically grown cells did not show the presence of cytochrome a, and only small and variable amounts of cytochrome b_1.

Barrett and Lemberg (29) maintained that with vigorous aeration B. subtils, Bacillus mycoides, and P. aeruginosa developed relatively small amounts of cytochrome a_2. The age of the culture (30,31) as well as the oxygen tension maintained (32,33) has also been shown to cause changes in the cytochrome composition of bacteria.

In recent years many investigators have reported purification of varying degrees for many of the individual bacterial cytochromes. Thus far cytochromes of the c type have proven to be the most easily isolated (34,35). Vernon and Kamen have succeeded in obtaining a purified cytochrome c from Rhodosprillum rubrum. The purification of cytochromes c_4 and c_5 from Azotobacter vinelandii has been reported by Tissieres (36,37). Cytochromes

of the c type have also been isolated from Pseudomonas fluorescens (38), P. aeurginosa (39,40), an aerobic halotolerant bacillus (41), Thiobacillus denitrificans (42), Pseudomonas denitrificans (43), and from an unidentified pseudomonad (44). A cytochrome with the properties of cytochrome c_1 has also been prepared from the above mentioned pseudomonad. A soluble cytochrome c_3 has been obtained from Desulfovibrio desulfuricans (45,46). Newton and Kamen have purified a cytochrome with spectroscopic absorption maxima in the reduced form at 552, 525, and 418 mμ. from Chromatium sp. (47,48). A highly purified form of cytochrome c from B. subtilis has also been prepared by several Japanese workers (49).

Horio et al. have succeeded in the purification of four kinds of respiratory components from P. auerginosa. Besides the c type cytochrome and the cytochrome oxidase already mentioned, a cytochrome with an alpha peak at 554 mμ. and a blue protein, which has been crystallized, have also been purified from this bacteria. The cytochrome oxidases of Micrococcus denitrificans (55) and A. vinelandii (56) have been partially purified. A soluble, autooxidizable and carbon monoxide binding pigment has been obtained from a strain of halotolerant bacteria (57). This pigment and the cytochrome oxidase of this bacteria are not identical. A cytochrome and a green pigment have been partially isolated from a sulfate reducing bacteria (58,59). The isolation and purification of a new heme protein from R. rubrum has recently been effected (60). A partial purification of a b type cytochrome was described by Birk et al. (61). Cytochromes similar to cytochrome b_1 have been isolated from M. denitrificans, P. denitrificans (43), and from an unidentified pseudomonad (44).

Those bacterial cytochromes which have thus far resisted all attempts at purification have usually been shown to be particulate, and the necessary extracting procedures for the individual cytochrome components have not as yet been worked out. The cytochromes of many bacteria have been shown to be associated with a particle obtained when the cellular unit was disrupted, either by sonic osillations, grinding, lysis or any of a number of methods employed to fragment the whole cell. Mycobacterium tuberculosis (62), P. vulgaris (63), A. vinelandii (67-69), and

A. peroxydans (70) were a few of the bacteria found to have their cytochrome complement integrated in some manner with a particle. These respiratory pigments form part of the particulate electron transport chain of these bacteria. In M. tuberculosis these cytochrome-containing particles displayed cytochrome oxidase, succinic dehydrogenase, L-malic dehydrogenase, DPNH dehydrogenase, and DPNH oxidase activity (62). In P. vulgaris, succinic oxidase and DPNH-cytochrome c reduçatase were observed in these particles. The terminal oxidase systems of S. marcesens (65), M. denitrificans, and A. vinelandii (68) were associated with a particle. Many of the other bacterial respiratory enzyme systems have also been shown to be particulate in nature.

Numerous enzymatic studies have been conducted with both the particulate cytochrome systems and with those cytochromes which have been solubilized. It is not the purpose of this review to cite all the work that has been done on the biological activities of the bacterial cytochromes. It is sufficient to state that the cytochromes have been shown to play a role in DPNH oxidation, succinate oxidation, nitrate reduction (17,72), reduction of colloidal sulfur (73), thiosulfate reduction (74), nitrite oxidation (75), sulfate reduction (76), and in the reduction of hydroxylamine (77). The enzymatic activities of cytochrome c reductase and cytochrome oxidase have been extensively studied in many bacteria.

It was mentioned earlier that B. subtilis had an absorption spectrum similar to that of yeast or mammalian tissues. For the most part, two techniques have been employed in the spectroscopic investigation of the cytochromes of this bacterium. Both Chance (5) and Smith (6) have utilized what is known as a difference spectrum in their studies of B. subtilis and other bacteria. In Smith's work the respiring bacteria were aerated and the change in optical density at a series of wavelengths from 380 mμ. to 650 mμ. were recorded with great sensitivity as the bacteria used up the oxygen in the solution and the pigments changed from the steady state oxidized to the reduced form. The optical density increments plotted against the wavelengths gave the difference spectra. She reported that B. subtilis has a cytochrome spectrum qualitatively similar to that of yeast and mammalian tissues.

Chance has studied the carbon monoxide compounds of the cytochrome oxidases of B. subtilis, A. pasteurianum, Staphylococcus albus, and Aerobacter aerogenes (5), and more recently those of seven additional bacteria (54). His information was obtained from direct recordings of the spectrum that represents the difference between the carbon monoxide compound and the cytochrome in the reduced form. The action spectra of the cytochrome oxidases of yeast, mammalian cells, and B. subtilis were found to be almost identical. Therefore it was concluded that the cytochrome oxidase of B. subtilis was of the type a_3, which is in agreement with the observations of Keilin and Hartree. It was shown that there was more cytochrome a_3 relative to cytochrome a in B. subtilis than in mammalian tissue or yeast. Smith investigated the cytochrome oxidases of several bacteria and reported that there was no cytochrome c oxidase activity in extracts of B. subtilis and several other bacteria (78). However, just recently Castor and Chance were able to observe oxidase activity for cytochrome a_3 in B. subtilis (54).

A second technique which has also been used to a great advantage in the study of the cytochromes of B. subtilis consisted of measuring the spectrum at the temperature of liquid nitrogen. Keilin and Hartree (79) noted the typical four banded absorption spectrum of yeast and mammalian tissue in cells of B. subtilis. Band c was stronger than band b at room temperature, but on cooling the cells in liquid air, the bands were intensified five to seven times. Band b was now stronger than band c, and a very narrow band appeared at 550 mμ. Recently this study was extended by Chaix and Petit (31). They made spectroscopic observation at varying times of two strains of B. subtilis at both room temperature and at the temperature of liquid nitrogen. For short periods of incubation, the strains exhibited alpha peaks corresponding to cytochromes a and b_1 at room temperature and a+b+y+z+c at the temperature of liquid nitrogen. The z band (553 mμ.) may be identical with the c_1 band. The y band (556-557 mμ.) has not been described previously. All bands except c_1 were found to be sensitive to oxygenation. For longer periods of time the strains exhibited an a+b+c spectrum at room temperature and at the temperature of liquid nitrogen, an identical but reinforced spectrum

which was devoid of the c_1 band. Chaix and Petit have also shown that it was possible to obtain a modification of the absorption spectrum due to hematin compounds of B. subtilis by simply varying the growth rate. The type of media chosen to grow the cells also affects the cytochrome composition.

It has been reported that cells of B. megaterium exhibit absorption maxima at 597, 558, and 525-530 mμ. when the cytochromes were reduced (80).

When cells of B. megaterium were treated with lysozyme in dilute phosphate buffer an extensive lysis occurred and only two microscopically detectable structural elements remained, the spherical empty ghosts and granules. If lysozyme treatment was performed in sucrose of appropriate concentration, the bacterial cell wall was depolymerized, but the rest of the cell remained as an intact structural unit, the protoplast. These protoplasts can be lysed by dilution of the solute, giving rise to the ghosts and granules obtained by lysis in phosphate buffer alone. Further studies have shown that the cytoplasmic membrane and the cell wall of B. megaterium were chemically quite different from each other (83). The wall contained large amounts diaminopimelic acid and hexosamine. These substances were absent from the membrane. The phosphorus content of the wall was higher than that of the membrane while its nitrogen content was lower. The cytoplasmic membrane can be characterized as a lipo-protein complex, perhaps containing smaller amounts of carbohydrate (glucose). Nucleic acids were essentially absent. It was highly probable that the complete cytochrome system of the cell was present in the membrane (82, 83). Experiments based on dye reduction showed that the enzymes responsible for all the succinate, DL-lactate, and alpha ketoglutarate oxidation, and 50% of the L-malate oxidation of the total lysate were located in the ghost fraction.

METHODS AND MATERIALS

Maintenance of stock cultures. Stock cultures of both *B. subtilis* and *B. megaterium* were obtained periodically from the Brigham Young University Department of Bacteriology. They were maintained by preparing new slants at various intervals depending upon the need of fresh slants to start the inoculum. It was found that new growth developed rapidly on an agar slant containing 0.8 g. nutrient broth, 1.5 g. bacto-agar and 0.5 g. yeast extract per 100 ml. of solution. The solution was heated to 100° C. to dissolve the agar and then poured in 5 ml. aliquots into test tubes which were stoppered with cotton plugs. The tubes were sterilized by autoclaving at 120° C. (15 lb. pressure) for twenty minutes. Transfers were made to these new tubes which were incubated at 37° C. for 24 hours. A very good growth was noted during this time. The cultures were stored in the refrigerator at 4°C. until transferred to new slants to maintain the culture.

Growth medium. The growth medium contained 10 g. bacto-peptone, 5 g. yeast extract, 2 g. K_2HPO_4, 1 g. NH_4Cl, and 0.1 g. $MgSO_4$ per liter of distilled water. The pH was adjusted to 7.0 and the solution sterilized by autoclaving at 120° C. (15 lb. pressure) for forty minutes. To each liter of the above solution was added 30 ml. of a 25% glucose solution which had been sterilized separately.

Preparation of inoculum. It was found that the best inoculum resulted when the bacteria were scraped from a fresh 24 hour slant. The inoculum medium contained 10 g. bacto-peptone, 2 g. yeast extract, and 5 g. glucose per 500 ml. of distilled water. To this was added 50 ml. of a 0.2 M. phosphate buffer solution at pH 7.0. Both solutions were sterilized before mixing. The inoculum was incubated at 37° C. for a 24 hour period.

Culturing the bacteria. The cells were grown aerobically in 20 liter bottles which contained 10 liters of growth medium. The medium was originally aerated by means of three tubes which contained spherical ends perforated with several small holes. Air was forced through these tubes and in this manner the medium was both aerated and agitated. Later one of the air leads was replaced by two dispersion stones connected to the lead with a glass "T" joint. The dispersion stones plus the remaining two spherical aerators provided excellent aeration of the medium. Foaming presented quite a problem. Several silicone antifoams were used, but in order to stop foaming completely, such large quantities of antifoam were required that it seemed to have a deleterious affect on the growth of the bacteria. The foaming problem was finally solved by providing an air outlet in the rubber stopper of the bottle. A rubber tube was connected to the outlet and the foam was washed down the drain. The pH of the medium became more acidic as growth continued, and it was necessary to check the pH periodically during the growth period. The pH was adjusted back to pH 7 with a 10% sodium hydroxide solution. Growth was allowed to continue for 36 hours at 30° C. which gave a good yield of cells. The cells were harvested and stored at -20° C. until they were used. The average yield of cells for B. subtilis was around 10 g. (wet weight) per liter of medium. The value for B. megaterium cells was around 6 g.

Equipment. A Beckman DU spectrophotometer was used for all enzyme assays, while a Beckman DK-2 recording spectrophotometer was used to obtain the spectra of the various preparations. A Raytheon Sonic Oscillator, 10 K.C., Model DF-101, was used to effect cell rupture.

Chemicals. Nutrient broth, bacto-peptone, yeast extract and bacto-agar were all obtained from Difco Laboratories, Detroit, Mich. Horse heart cytochrome c, reduced diphospho-pyridine nucleotide, tris (hydroxymethyl) aminomethane, desoxy-ribonuclease, and lysozyme were purchased from the Sigma Chemical Co., St. Louis, Mo. Bovine plasma albumin, lipase, trypsin, pancreatin, pepsin, and papain, were obtained from the Nutritional Biochemical Corp., Cleveland, Ohio. The remaining chemicals

were obtained from commercially available sources and were of reagent or C. P. grade. Ion low water was prepared by passing distilled water through a column filled with Amberlite MB-3 resin.

<u>Assay system for diaphorase.</u> The assay system contained 0.3 ml. of 0.2 M. phosphate buffer, pH 7.0, 0.1 ml. of 0.0012 M. DPIP,* 0.05 ml. of 0.01 M. DPNH,* 2.45 ml. of ion low water, and 0.1 ml. of a suitable dilution of the fraction to be tested. The activity was assayed by following the decrease in optical density at 590 mμ. The reaction was started by adding the enzyme preparation and then readings were taken either every 15 or 30 seconds depending upon the activity observed. The decrease in optical density in the first five minutes per mg. of total protein was taken as the specific activity. One unit of enzyme activity was equivalent to a fall in optical density of 1.00 per five minutes.

<u>Assay system for cytochrome c reductase.</u> The activity was determined by observing the optical density increase at 550 mμ. The assay system contained 2.15 ml. of ion low water, 0.3 ml. of 0.2 M. phosphate buffer, pH 7.0, 0.3 ml. of 0.01 M. potassium cyanide, 0.15 ml. of a 1% horse heart cytochrome <u>c</u> solution, and 0.1 ml. of a diluted enzyme preparation. To this mixture was added 0.1 ml. of a 0.01 M. DPNH solution and readings were taken at 15 or 30 second intervals for a period of five minutes. Both the specific activity and the unit of enzyme activity were defined as described for the assay of diaphorase except that the increase in optical density was measured.

<u>Assay system for cytochrome c oxidase.</u> The assay system contained 2.45 ml. of ion low water, 0.3 ml. of 0.2 M. phosphate buffer, pH 7.0., and 0.15 ml. of a 1% horse heart cytochrome <u>c</u> solution. The cytochrome <u>c</u> was reduced by the careful addition

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*The following abbreviations will be used in this thesis: DPNH for diphosphopyridine nucleotide and DPIP for 2, 6-dichlorophenol indophenol.

of sodium dithionate until the cytochrome was 80-90% reduced. The oxidase activity was assayed by following the decrease in optical density after 0.1 ml. of a suitable dilution of the enzyme had been added. Measurements were made at 550 mμ. and the readings were taken every 15 or 30 seconds for a period of five minutes. Both the specific activity and the unit of enzyme activity were as defined for diaphorase.

Assay system for DPNH oxidase. DPNH oxidase was determined by observing the optical density decrease at 340 mμ. The assay system contained 2.5 ml. of ion low water, 0.3 ml. of 0.2 M. phosphate buffer, pH 7.0, and 0.1 ml. of a diluted enzyme preparation. The reaction was started by adding 0.1 ml. of 0.01 M. DPNH. Readings were taken every 15 or 30 seconds for a period of five minutes. The same definition of specific activity and unit of enzyme activity was used as before.

Assay system for succinic dehydrogenase. To 10 ml. of a 0.01 M. solution of phenazine methosulfate was added the following: 2.0 ml. of 0.2 M. phosphate buffer, pH 7.0, 0.2 ml. of 0.1 M. potassium cyanide, and enough ion low water to make the total volume 20 ml. In each assay, 2.0 ml. of this solution were used. The decrease in optical density was measured at 430 mμ. after 0.1 ml. a diluted fraction as well as 0.2 ml. of a 0.1 M. succinate solution had been added to the solution. The reaction was performed ~~aerobi~~cally.

General procedure for sonorating cells. The harvested cells usually contained large amounts of contaminating color from the media. Therefore, it was profitable at this point to wash the cells two or three times in ion low water. As a general practice the cells were washed until the resulting supernatant fluid from the centrifuged cells appeared almost color free, following which from 10 to 15 g. of cells were suspended in a total volume of 50 ml. of M/15 phosphate buffer, pH 7.0. The suspension was homogenized in either a glass homogenizer or Waring blendor; the cells were placed in the cup of the sonic osillator and sonoration was allowed to continue for a period of 30 minutes.

The suspension was centrifuged at 60,000 x g. The resulting supernatant fluid was the starting material for the majority of the fractionation procedures which will now be described.

Enzyme Digestions. The material to be digested was suspended in M/15 phosphate buffer, pH 7.0. The dry enzyme powder was added to the solution until a concentration of either 1 or 2 mg. per ml. of solution was obtained. Two methods have been used, in the first the enzyme was left in contact with the substrate overnight at room temperature and in the second the digestion was allowed to continue for 30 minutes at 37° C.

Preparation of acetone powders. Ten grams of wet cells were added to 100 ml. of cold acetone (-14° C.), and allowed to set for 10 to 15 minutes in an ice-salt bath. In order to obtain an even suspension it was sometimes necessary to homogenize the suspension in a Waring blendor. The suspension was filtered onto a Buchner funnel, the powder washed several times with cold acetone (and in some instances with cold ether), and the powder placed upon a large sheet of filter paper for drying. It was then stored at a -14° C.

Detergents as extracting agents. The following detergents have been used: Triton X-100, Wisk, sodium cholate, digitonin, and Pluornic L-44. A 1% solution was used as the extracting agent. The detergent was either added to the solution directly, or else the particulate matter was precipitated with ammonium sulfate and then taken up in a 1% solution of the detergent. The length of the extraction varied greatly.

Ghosts preparation. Ten grams of wet cells were suspended in 250 ml. of M/50 phosphate buffer, pH 7.0. To this suspension was added 82.5 ml. of a 2 M. sucrose solution, 3.75 ml. of a 1 M. sodium chloride solution, and 100 mg. of crystalline lysozyme. Digestion was allowed to continue until protoplast formation was complete. This usually took from 30 to 120 minutes. The protoplasts were quickly resuspended in 50 ml. of ion low water containing 0.2 mg. of desoxyribonuclease. Cell rupture was

completed in about 30 minutes. The ghosts were then centrifuged for 30 minutes at 25,000 x g., and then washed three times with ion low water. After each washing the ghosts are spun down at 28,000 x g.

EXPERIMENTAL RESULTS

Cell rupture. Several methods were used in an effort to obtain a cell free extract rich in cytochromes. Each of these methods is described briefly and an appraisal of the effectiveness of each method will also be presented.

(1) Freezing and Thawing

Ten grams of cells were suspended in 50 ml. of M/15 phosphate buffer, pH 7.0. The suspension was frozen and thawed three times and then centrifuged. The supernatant liquid was tested for protein content by means of the Biuret reaction and also spectroscopically for cytochromes. The suppernatant liquid contained only 2.7 mg. of protein per ml. of solution, and was devoid of cytochromes as determined spectroscopically.

(2) Osmotic Shock

Cells were suspended in 2 M. sucrose for several minutes and then centrifuged. The collected cells were then quickly placed in ion low water. Some rupture was effected, but the cytochromes were not released into solution by this method.

(3) French Cell

Ten grams of cells were suspended in 20 ml. of buffer, frozen in the chamber of a French cell, and the frozen cells were then forced through the small orifice. The protein concentration of the supernatant fluid after centrifugation was 12.7 mg. per ml. of solution. Even though excellent rupture was obtained by this method, the cytochromes were evidently bound in some combination so that rupture of the cell wall did not liberate them from the particulate matter.

(4) Sonic Oscillation

Sonic rupture was by far the most effective method of any of those tried. Cell rupture took place due to sonic shocks, and upon repeated exposure the cell wall and the cell membrane were fragmented. It appeared that fragmentation of the cell membrane released the particles containing the cytochrome components of the bacteria.

(5) Grinding With Alumina

Equal amounts of cells and alumina were mixed together and then frozen. The mixture was ground in a mortar while thawing took place. This process was repeated three to five times. This was a somewhat tedious method of effecting cell rupture, but nevertheless excellent rupture was obtained. Again it appeared that some fragmentation of the cell membrane had taken place. The ground paste was extracted with M/15 buffer, and the suspension centrifuged differentially to rid the mixture of alumina powder first, and then cell debris. The supernatant fluid displayed a typical cytochrome difference spectrum. It was probably the fragmented cell membranes that indicated the presence of cytochromes in the extract.

(6) Acetone powders were prepared as described in the section on methods and materials and extracted with borate and citrate buffers of varying strengths. These buffers failed to extract a cytochrome component. An 8 M. urea extraction of the powder resulted in rupture and fragmentation. Particulate cytochromes were observed spectroscopically in the supernatant fluid. The cell residue from the urea extract was sonorated and this resulted in an additional quantity of the cytochrome containing particles being liberated. This indicated that the urea rupture was incomplete.

(7) Cells treated with lysozyme could readily be ruptured. In fact if rupture of the cells was all that was necessary to release the cytochromes into solution this would be the method of choice to effect cell rupture. However, the rupture was attended by the release of large amounts of extraneous material which made spectroscopic investigation of the various fraction impossible.

Difference spectra of sonorated cells. A complete determination of the cytochrome composition of *B. megaterium* or of *B. subtilis* was not accomplished. One reason for this was the marked difference in the cytochrome composition from one batch of cells to the next. As an example, many cells seemed to be devoid of a peak between 595 and 605 mμ., while other cells exhibited a very pronounced peak in this region. There was also a great deal of variation in the cytochrome *c* content of different batches of cells.

A typical absorption spectrum of a *B. megaterium* extract is given in Figure 1, showing quite a pronounced peak between 595 and 605 mμ. A strong absorption was also found in the region of 545 to 565 mμ. In most cells this was represented by a symmetrical peak with a maximum around 557 mμ. There was some variation in both the intensity and the shape of this absorption band. Occasionally an adjoining peak was observed at 550 mμ. which corresponded to a *c* type cytochrome. Whenever this peak was present, an excellent yield of cytochrome *c* was obtained upon further purification. Figure 2 shows the spectrum of a batch of cells with a high cytochrome *c* content. By subsequent fractionations it could be shown that the peak routinely observed at 557 mμ. was probably due to the presence of a cytochrome *b*, some cytochrome *c*, and very likely some cytochrome c_1. It was possible to physically separate the cytochrome *c* from the other cytochromes by a controlled enzymatic digestion with lipase.

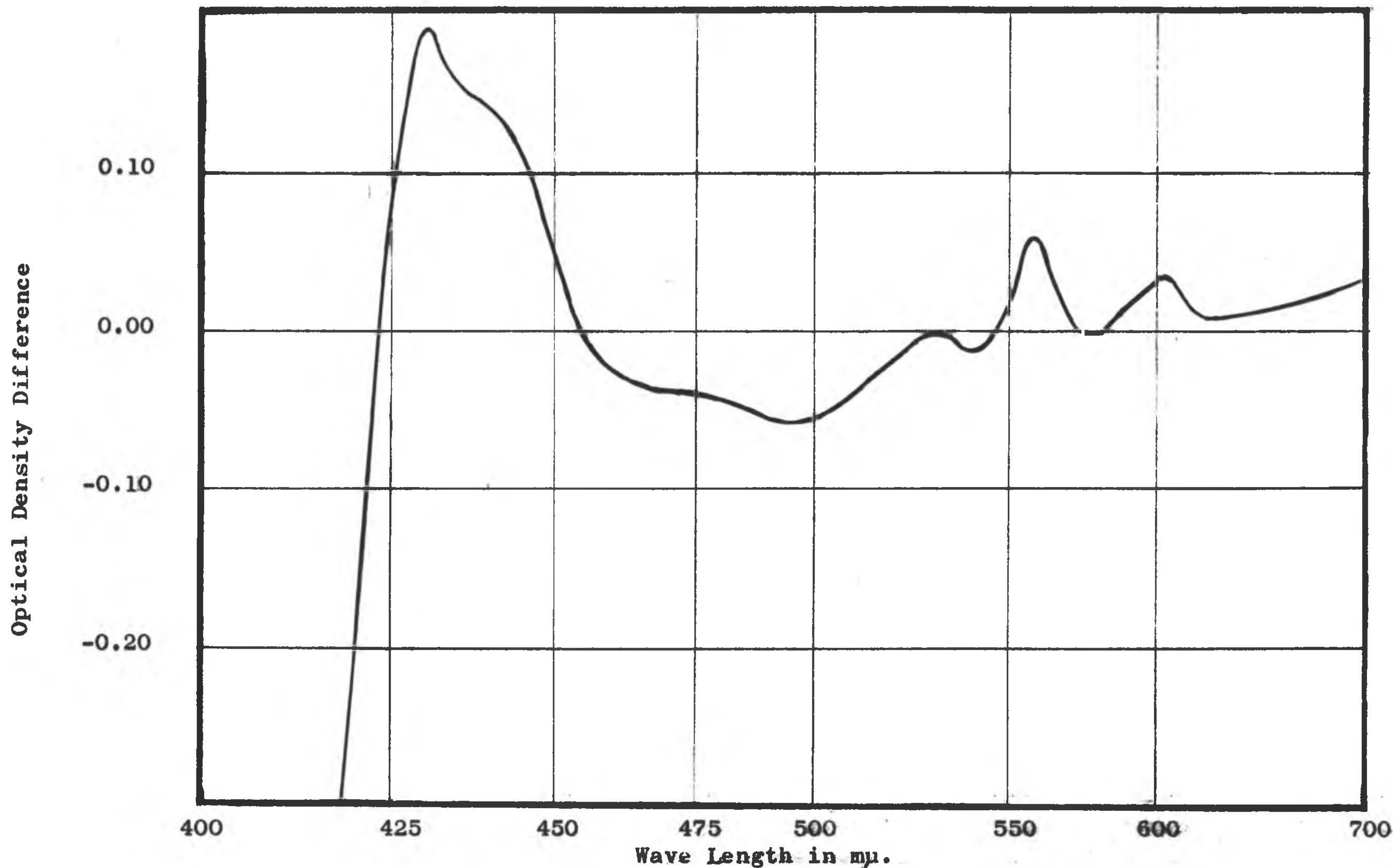

Figure 1. The difference spectrum of soronated B. *megaterium* cells having a low cytochrome c content. This curve represents a Beckman DK-2 trace. The sample cell contained the extract with dithionate present, while the reference cell contained the non-treated extract.

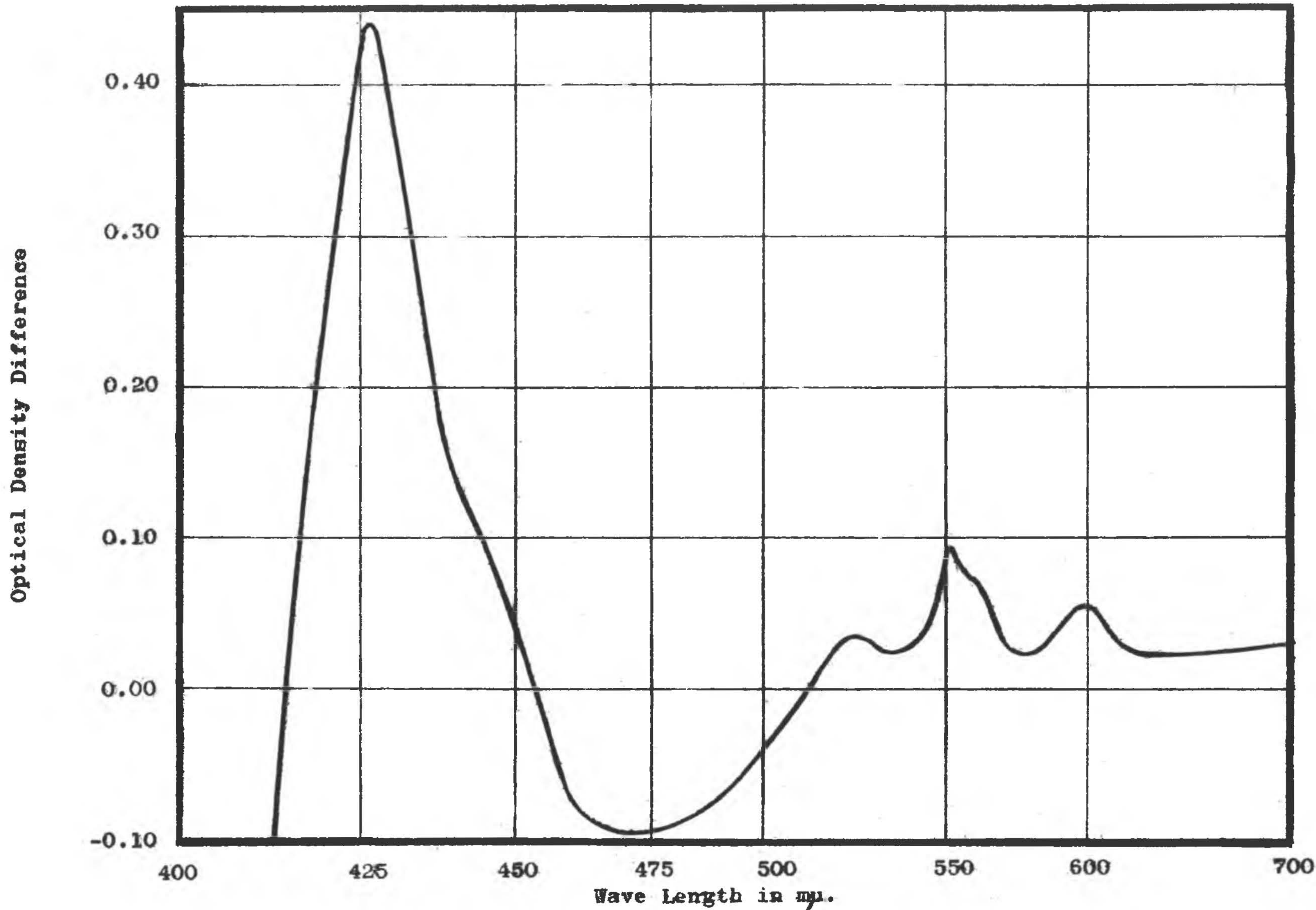

Figure 2. The difference spectrum of sonorated B. megaterium cells having a high cytochrome c content, obtained as in Fig. 1.

Ammonium sulfate fractionation. Ammonium sulfate was used in the early stages of purification mainly as a method of concentrating the particulate matter obtained from sonoration. In almost every preparation the majority of the cytochrome containing particles were precipitated in the 0 to 20 fraction. A few of the particles were not precipitated in the 0 to 20 fraction, but rather in the 20 to 40 fraction. At this concentration (40 g. per 100 ml.) of ammonium sulfate, almost all the color had precipitated from the solution and upon spectroscopic analysis no indication of cytochromes was obtained. A limited purification resulted from such a fractionation, since some of the extraneous matter was separated from the particles bearing the cytochromes. The 0 to 20 fraction was dissolved in a 1% trition solution, and a second ammonium sulfate fractionation was conducted. Two main fractions were collected. One fraction came to the top in the presence of 20 g. of salt per 100 ml. of solution. This fraction was salted out of solution very sharply. Recent experiments seem to indicate that the triton was extracting a portion of the particles, and when ammonium sulfate was added, the triton was salted out of solution. Additional ammonium sulfate was added to the remaining supernatant fluid, until a concentration of 40 g. per 100 ml. was obtained. At this concentration a precipitate was obtained which settled to the bottom when centrifuged. The amount of particulate matter which went with each fraction varied greatly. In some cases almost all of the particles were found in the 0-20 fraction, and in every case the majority of the particles were found in this fraction. Both fractions displayed a representative cytochrome spectrum. There was some difference in the spectra of the two fractions but, this was mainly in the intensity of the different peaks rather than in the location of the peaks. Here again, some variety was noted in extracts obtained from different batches of cells.

Ultracentrifugation. The ultracentrifuge has provided a very usefull tool for concentrating the cytochrome containing particles. A differential centrifugation of the sonorated cell suspension was used routinely in preparing the particles for further purification. After most of the cell debris had been

spun down at 18,000 x g., the particulate matter containing the cytochromes remained in solution and was not precipitated even when the centrifugal force was increased to 60,000 x g. This did, however, yield a clearer fraction. Therefore, as a standard procedure, after sonoration the suspension was centrifuged at 60,000 x g. If the above supernatant fluid was subjected to a centrifugal field above 100,000 x g. a concentration of color was noted in the bottom of the centrifuge tube. If this fraction was centrifuged at 144,000 x g. for an hour or longer, a pellet collected in the bottom of the tube. A strong concentration of color was also present directly above the pellet. Both the pellet and the concentrated color layer were rich in the cytochrome bearing particles. The size of the pellet and the amount of concentrated color depended upon the length of time the fraction was centrifuged.

The supernatant fluid above the concentrated color layer was first removed with a hypodermic needle, and the concentrated color layer from the several centrifuge tubes was combined. The remaining pellets were then suspended in buffer and sonorated for two minutes. Spectroscopic measurements of the various fractions could not be made directly because of the large amounts of extraneous color present. It was necessary to compare the reduced forms of the cytochromes in the various fractions to the oxidized forms. Such a spectrum is referred to as a difference spectrum. The cytochromes were reduced with dithionate and oxidized with ferricyanide. The presence of ferricyanide in the reference cell made it impossible to measure the bands in the Soret region. A comparison of the sonic extracts of B. subtilis and B. megaterium usually showed that the B. megaterium had the superior cytochrome content. This was particularly true of the type a cytochrome. For this reason most of the spectroscopic studies were conducted with extracts obtained from cells of B. megaterium rather than from B. subtilis.

In one batch of cells of B. megaterium, a very interesting absorption pattern was obtained. Sharp peaks were observed at 600 and 550 mμ. with a pronounced hump on the 550 peak at 600 mμ. The spectrum of the concentrated color layer above the pellet

obtained from these cells is contained in Figure 3. Figures 4, 5, and 6 also show spectra of other concentrated color layers derived from extracts of different batches of cells. A great deal of variety can be noted in the cytochrome content of the different extracts. While Figure 3 has a very definite peak at 550 mμ., Figures 5 and 6 show this peak to be missing. Figure 4 is devoid of the peak at 557 mμ. and instead peaks strongly at 550 mμ. Figure 5 has an absorption maximum at 557 mμ. rather than at 555 mμ. as is seen in Figure 6. The location of the peak at 555 mμ. in this case indicated the possible presence of cytochrome $\underline{c}_1$.

Centrifugation at 144,000 x g. for two or more hours resulted in a concentration of the cytochrome containing particles. Some purification resulted due to the fact that the lighter material remained in solution, while the heavier particles were concentrated in either the pellet or the color layer above the pellet. A comparison of the relative amounts of the cytochrome containing particle concentrated in each of these two fractions will be made in another section of this thesis.

Enzymatic disgestions. In recent experiments the use of hydrolytic enzymes has made possible a partial purification of cytochrome c. Different digestions have also produced a limited purification of some of the other cytochromes, and there has been some indication that type a cytochromes as well as cyrochrome $\underline{c}_1$ might be separated from the particle by the proper choice of a hydrolytic enzyme, as well as suitable digestion conditions. It must be pointed out that the hydrolytic enzymes were most useful in liberating cytochrome c, and only a partial liberation of the other cytochromes was affected. In fact if digestion was allowed to continue for any length of time cytochromes of both the a and b type were completely destroyed.

The enzymes used in these studies included pancreatin, ribonuclease, lysozyme, pepsin, lipase, trypsin, and papain. In the early experiments the digestions were allowed to continue for 30 minutes at 37° C. However, when a longer period of time was used it was possible to separate cytochrome c from the other cytochromes of the particle. The two enzymes which effected this

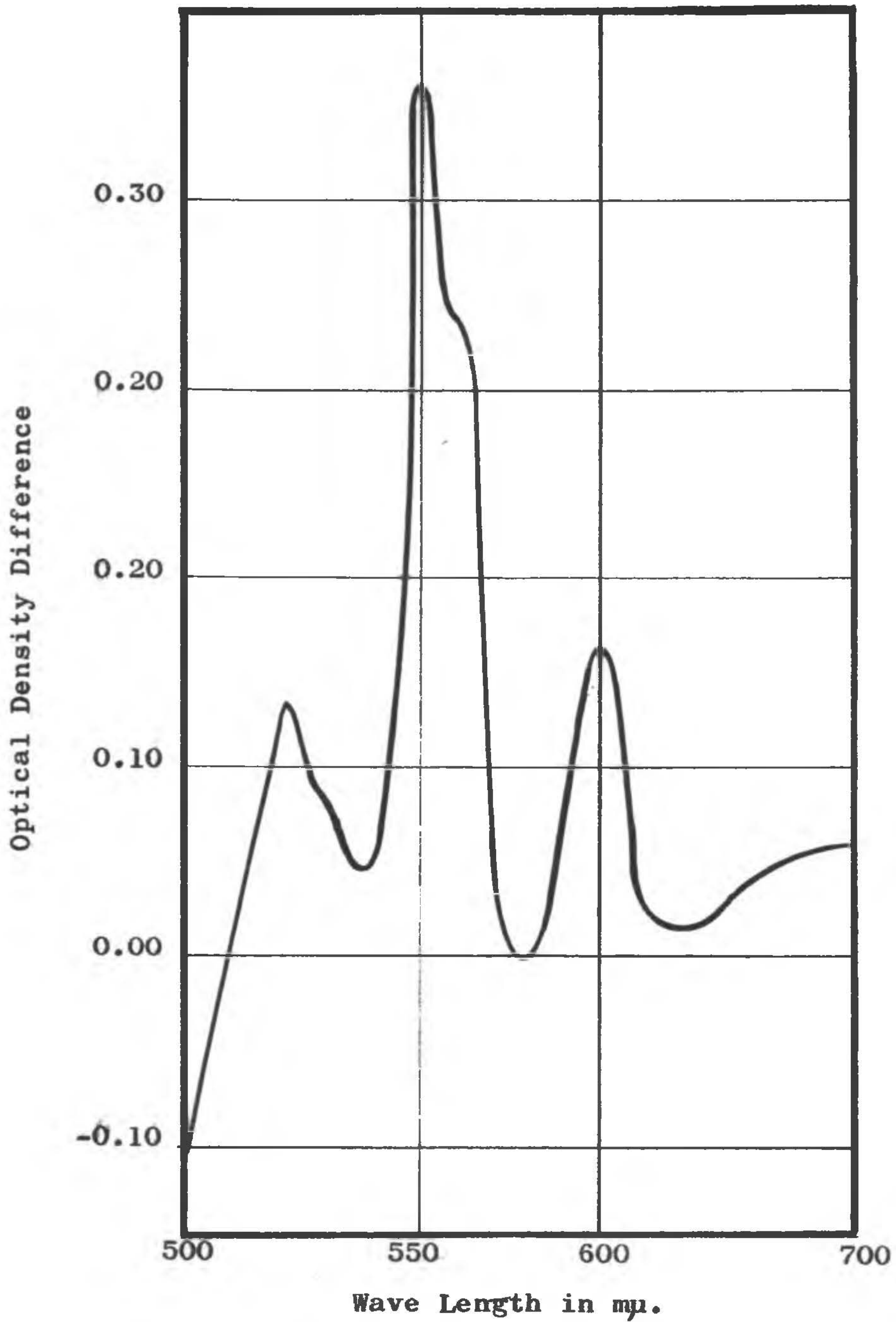

Figure 3. The difference spectrum of the concentrated color layer obtained from B. megaterium cells with a high cytochrome c content. This trace was obtained in the same way as in Fig. 1, except that the reference cell was oxidized completely with ferricyanide.

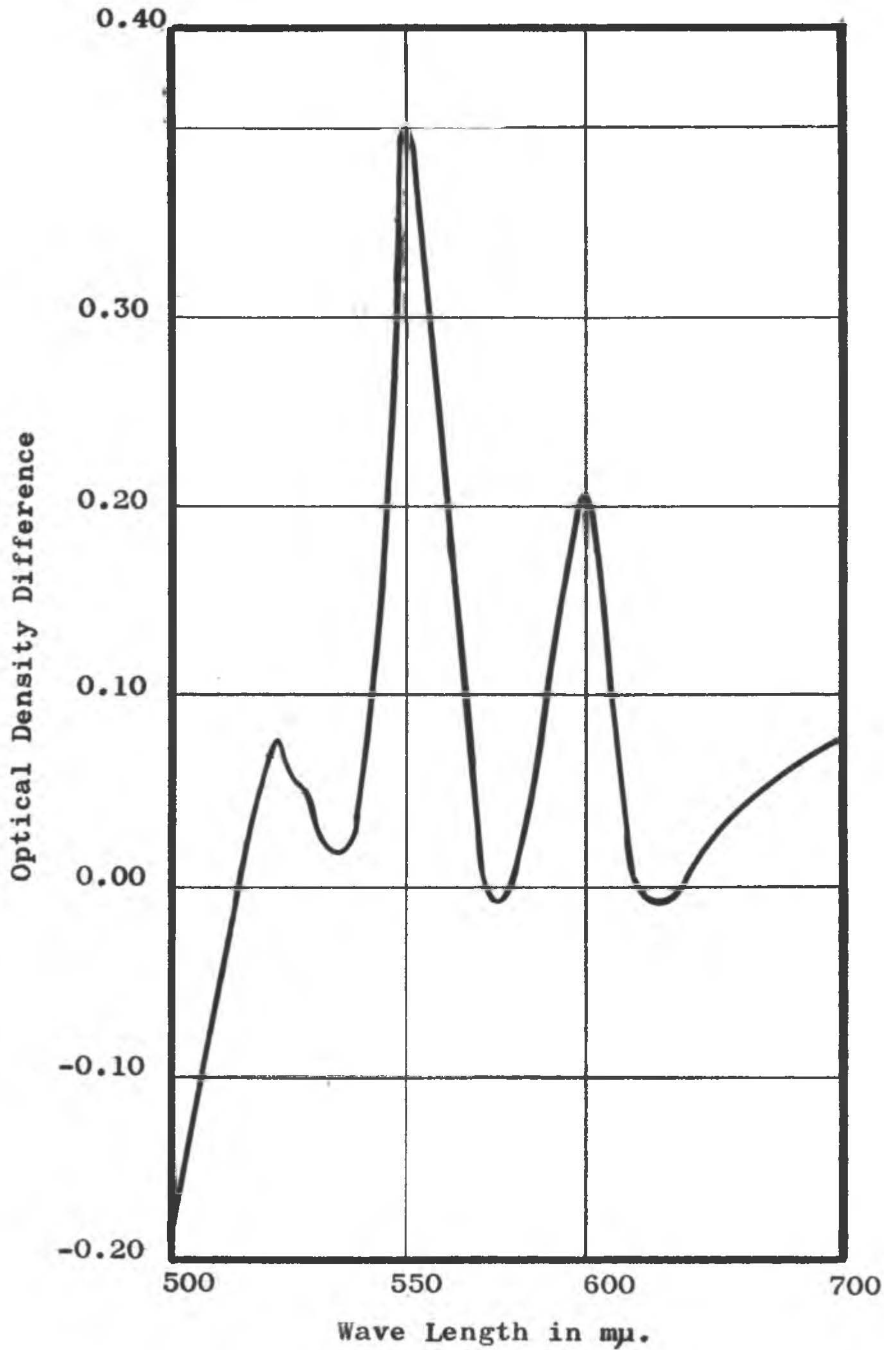

Figure 4. The difference spectrum of the concentrated color layer obtained from B. megaterium cells which were relatively low in the cytochrome b component. This trace was obtained as in Fig. 3.

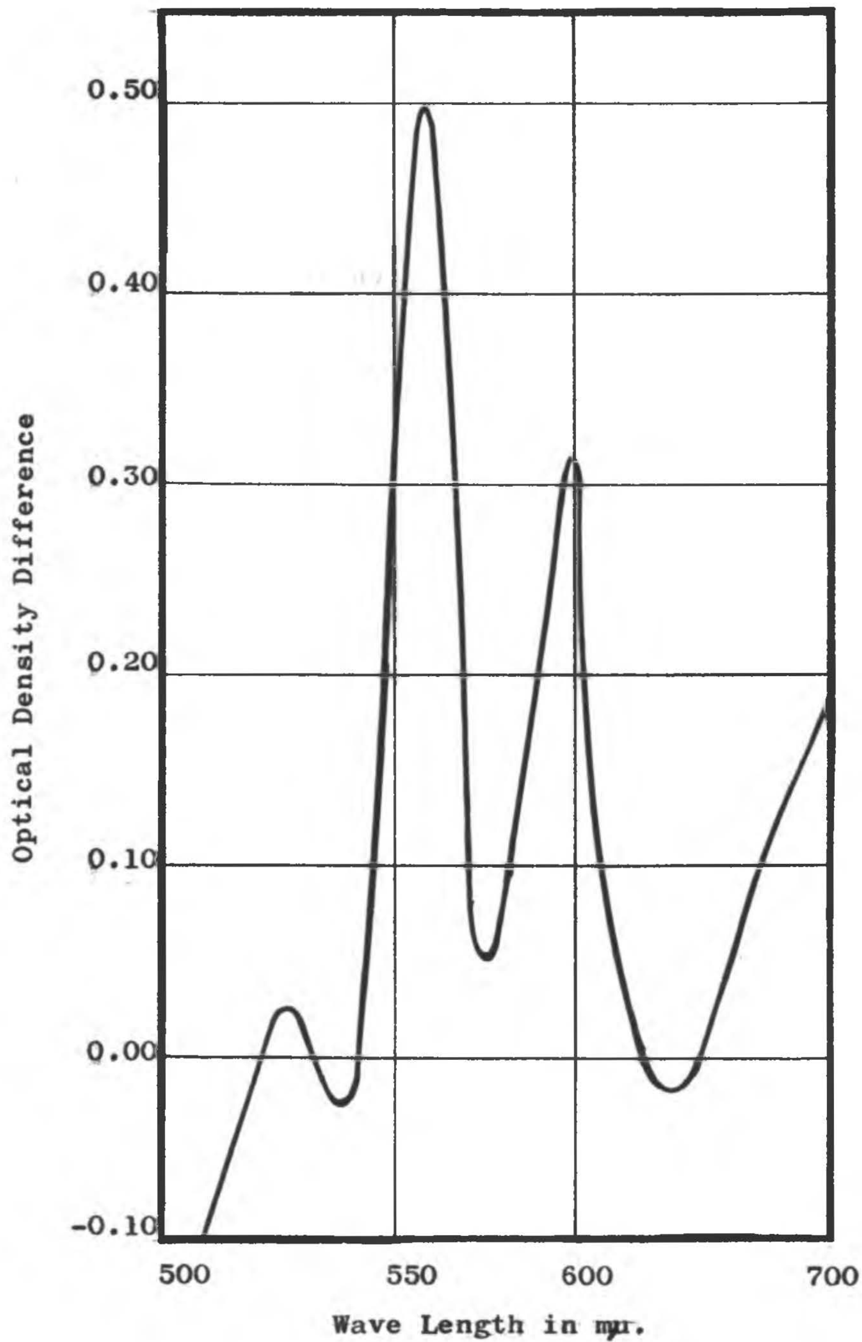

Figure 5. The difference spectrum of the concentrated color layer of B. megaterium cells which were low in cytochrome c content. This trace was obtained in the same way as Fig. 3.

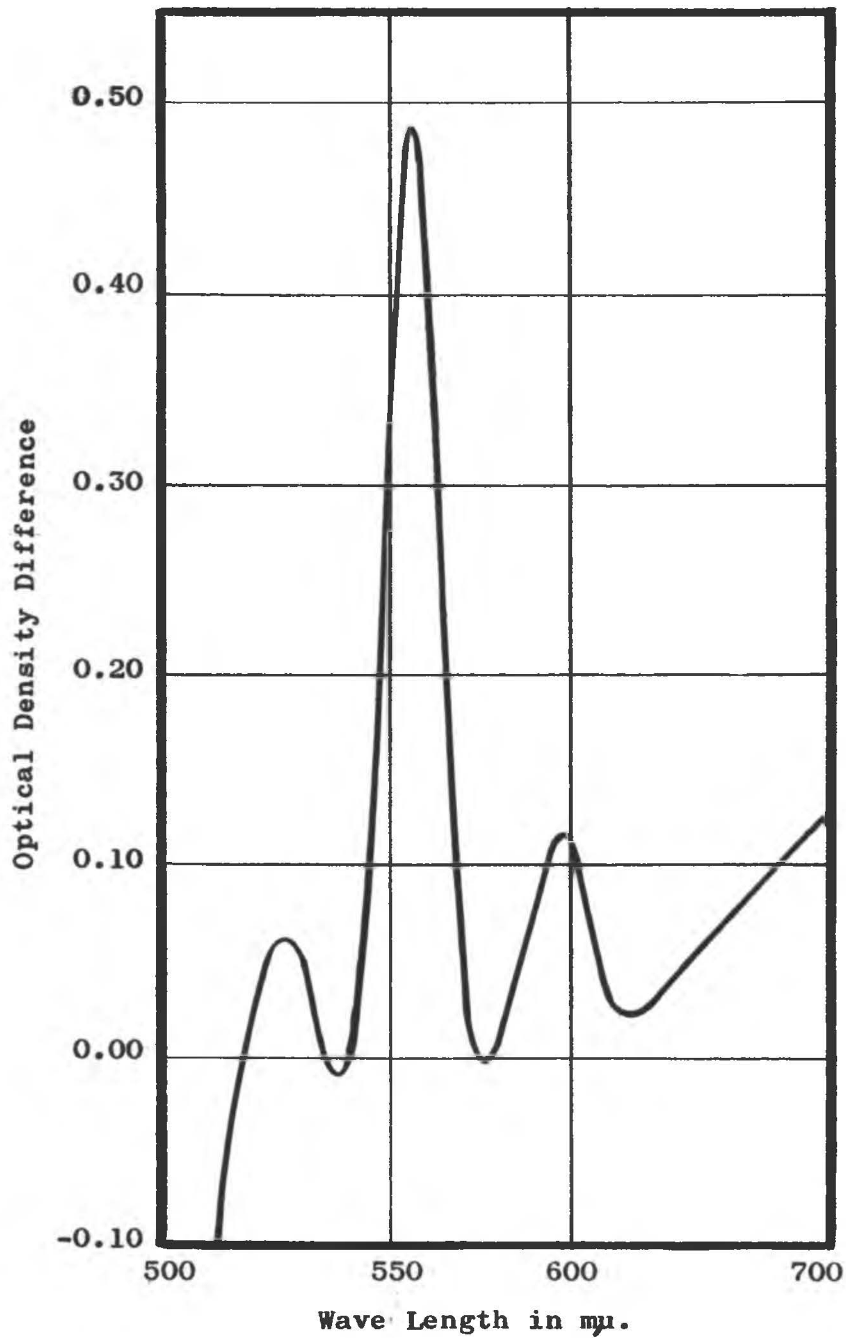

Figure 6. The difference spectrum of the concentrated color layer obtained from B. megaterium cells with a relatively low content of type a cytochrome. This trace was obtained in the same way as Fig. 3.

release were lipase and pancreatin. A more detailed description of the partial purification of cytochrome c will be presented in another section.

Attempts to further fragment the particulate matter. After the cytochrome containing particles were obtained, several attempts to further fragment them were made. Several detergents were used in an effort to either fragment the particle or extract the cytochromes from the particles. Of all the detergents used, Triton X-100 had the most profound effect upon the particles. An extraction resulted which was much less colored, but still displayed a similar cytochrome composition to that of the original concentrated color layer. No net purification was obtained with any of the other detergents. In one series of experiments, the cells were treated with the different detergents, and then an attempt to extract the cytochromes with 0.12 M. potassium chloride was tried. The salt solution was totally ineffective in extracting the cytochromes. As has been mentioned before, it was possible to obtain some fragmentation of the particle by the use of different hydrolytic enzymes. The enzymatic digestion of the particles with lipase to liberate cytochrome c was greatly aided by the prior treatment of the particle with triton. In fact very little cytochrome c was released unless this step was carried out.

In the many experiments using organic solvents as extracting agent, there was never any indication that any cytochrome had been extracted with these solvents. The same results were true of an extraction with warm trichloroacetic acid. Likewise an extraction with a sodium hydroxide solution of pH 10 was unsuccessful. Many experiments were carried out in an effort to extract the cytochromes from an acetone powder of the whole cell. The only agent which even showed possibilities was urea. An extract obtained with urea resembled the fraction obtained when the acetone powder was sonorated. However, these extracts were not superior to those obtained when whole cells were sonorated. Borate and citrate buffers were also used as extracting agents, but without success.

Several attempts were made to prepare bacterial ghosts by treating the cells with lysozyme. It was difficult to obtain enough clean ghosts to conduct spectroscopic examinations. For this reason and because of the lengthy processed involved in their production, it was decided to routinely sonorate the cells and collect the fragmented cell membranes by centrifugation.

Partial purification of cytochrome c. Many of the more common methods of extracting cytochromes, such as warm trichloroacetic acid, organic solvents, detergents, etc., from bacterial sources proved totally ineffective in causing a similar liberation of the cytochrome of either B. subtilis or B. megaterium indicating the cytochrome c was not in the cytoplasm. The cell membrane has been characterized as a lipo-protein complex, and it was thought that if this complex could be disrupted in some manner it might result in the liberation of one or more of the cytochrome components. It was found that enzymatic digestions would do this and a partial purification of cytochrome c resulted.

Forty grams of whole cells were suspended in 300 to 400 ml. of ion low water. The suspension was homogenized in a Waring blendor for 30 seconds. The washed cells were centrifuged at 12,000 x g., and then resuspended in sufficient M/20 phosphate buffer, pH 7, so that the total volume of cells plus buffer was 200 ml. The cell slurry was sonorated in 50 ml. batches for 30 minutes. The supernatant fluid is saved and the debris discarded. Forty grams of ammonium sulfate were added for every 100 ml. of solution, and the fraction was centrifuged at 25,000 x g. The precipitate was redissolved in a 1% triton solution. At this point approximately 150 ml. of 1% triton solution were required to suspend the precipitated particles. The particles were allowed to stand in the detergent for a period of 8 to 12 hours. The solution was then centrifuged and the precipitated material discarded after which a second ammonium sulfate fractionation was preformed. In this case two fractions were collected, the 0-20 fraction and the 20-40 fraction, representing the points where 20 and 40 g. of ammonium sulfate had been added for each 100 ml. of solution. Nearly all the cytochrome was contained in these two fractions. The 0-20 fraction came to the top when

the solution was centrifuged. The precipitated material was carefully collected by filtering the supernatant liquid through filter paper. The portion which remained in the tube and that which was on the filter paper were combined. Both this fraction and the 20-40 fraction which settled to the bottom when centrifuged were resuspended in 50 ml. of phosphate buffer, pH 7. Lipase was added to these two fractions until the concentration reached 2 mg. per ml., and digestion was conducted at room temperature for a period of from 8 to 12 hours. The digested mixture was centrifuged and the precipitate discarded. Fifty grams of ammonium sulfate were added to each 100 ml. of the supernatant fluid. The precipitate was discarded and trichloroacetic acid was added to the solution until 1 ml. of 0.5 M. acid had been added for each 10 ml. of the solution. The precipitation of the cytochrome was allowed to continue at 0° C. for 30 to 60 minutes. The solution was then centrifuged and the precipitate was dissolved in about 5 ml. of buffer. It was usually necessary to centrifuge the dissolved cytochromes to rid the solution of denatured protein. The spectrum was then taken of the sample. A liberation of cytochrome c could only be effected from those cells whose original sonic extract contained a relatively high content of cytochrome c, as could be evidenced by a sharp peak at 550 mu. when a spectrum of the extract was examined.

A simpler method of concentrating the cytochromes prior to conducting the enzymatic digestion has been developed. Instead of performing an ammonium sulfate fractionation, the sonorated material was differentially centrifuged. The extract was finally centrifuged at 144,000 x g. for a period of 2 to 4 hours. The pellet and the concentrated color layer were combined and sonorated for two minutes in order to resuspend them in solution. Triton was then added directly to the solution or else a 1% triton solution was used as the original resuspension medium. From this point on the purification steps were identical to those already described.

Figure 7 shows a representative spectrum of the partially purified cytochrome c from B. megaterium. A spectrum of the partially purified cytochrome c from B. subtilis is given in Fig. 8.

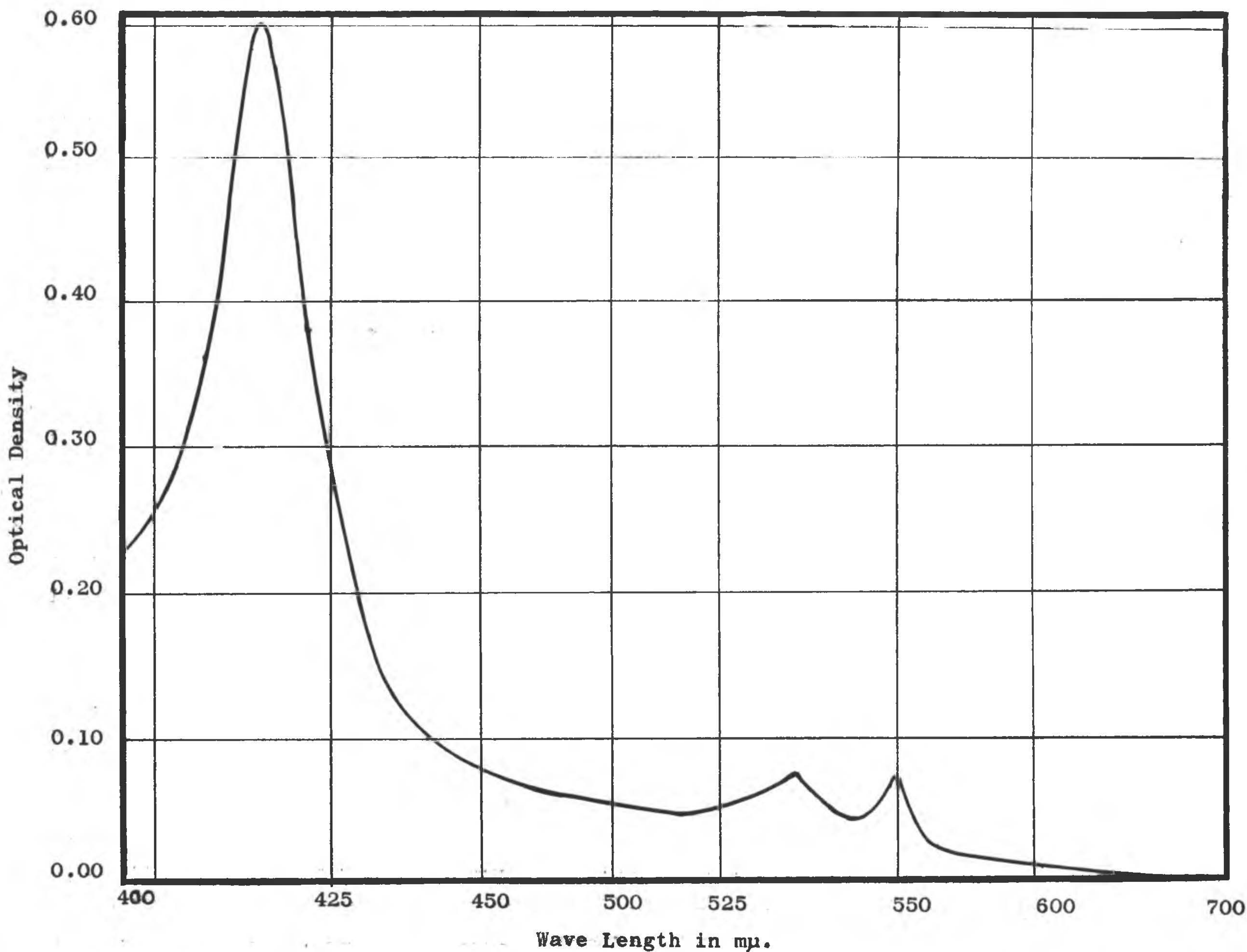

Figure 7. The reduced, direct spectrum of the partially purified cytochrome c obtained from B. megaterium.

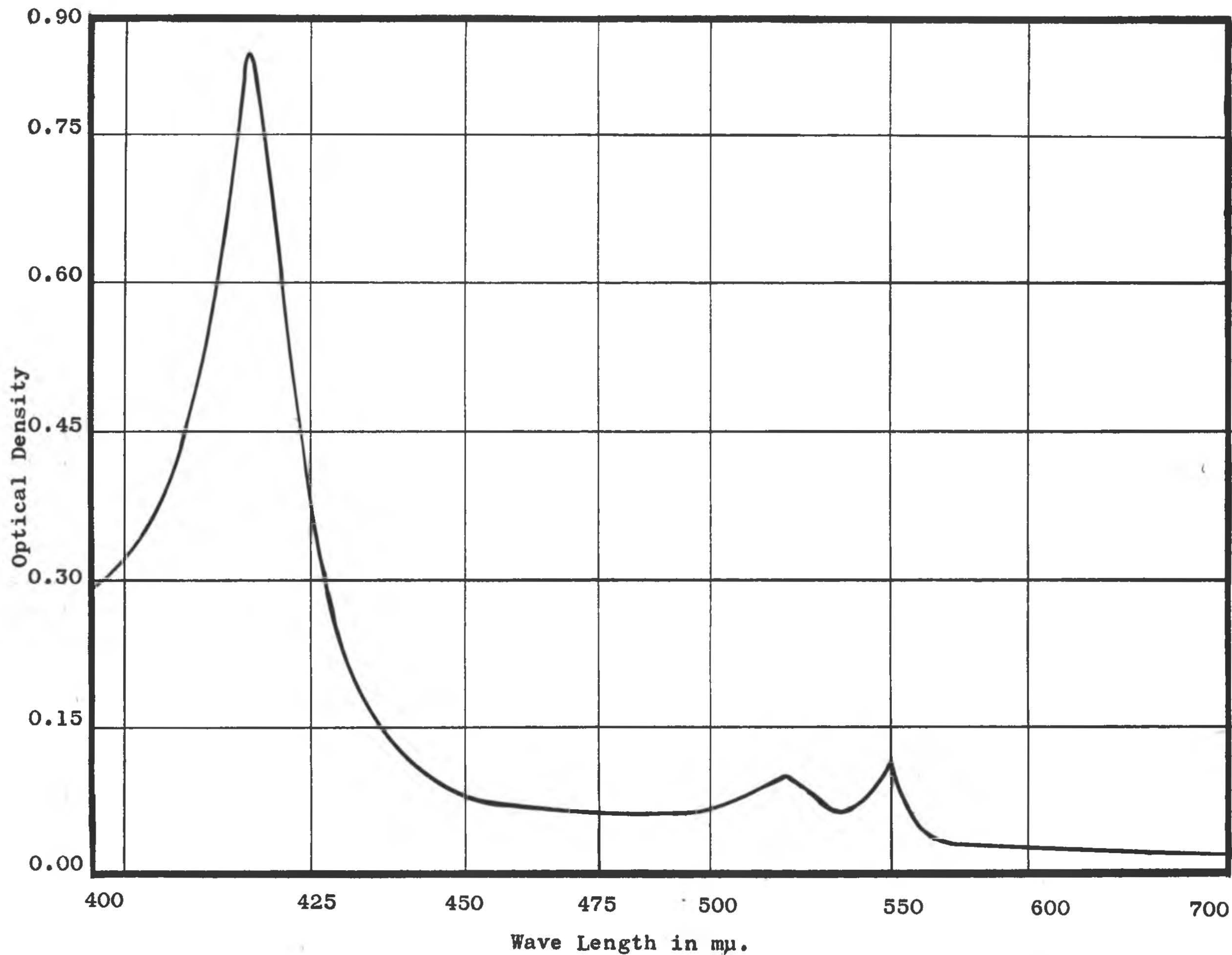

Figure 8. The reduced, direct spectrum of the partially purified cytochrome *c* obtained from *B. subtilis*.

Both spectra show an alpha absorption maximum at 550 mμ. The beta peak is located at 520 mμ. The strong Soret peak at 415 mμ. shifted to 408 mμ. when the pigment was oxidized. The exact location of these peaks were confirmed by careful measurements with a DU spectrophotometer.

Enzymatic Studies. Six fractions of a sonorated suspension of *B. megaterium* cells were obtained by differential centrifugation of the suspension. The original cell suspension after sonoration is referred to as Fraction I. This fraction was centrifuged at 60,000 x g. for 30 minutes. The supernatant fluid (Fraction II) was recentrifuged at 144,000 x g. for 3 hours. The upper layer (Fraction III), comprising most of the supernatant fluid was separated from the concentrated color layer (Fraction V) and the pellet (Fraction VI). Fraction IV was composed of the small amount of supernatant fluid which was directly above the concentrated color layer. Fraction I was a turbid gray, and was quite viscous due to the presence of deoxyribonucleic acid. The solution in Fraction II was a deep red-yellow. Fraction III was a straw yellow. Fraction IV was similar to Fraction II. Fraction V was an intense red-green color. The pellet was a dark red, but when it was resuspended it resembled Fraction V.

The protein concentration of the various fractions is given in Table I.

Table I

Protein Content of the Various Fractions

Fraction	Protein Concentration mg./ml.	Total Volume ml.	Total Protein mg.	% Recovery of Total Protein
I	28	200	5600	100.0
II	22	150	3300	58.9
III	12	110	1320	23.6
IV	28	30	840	5.0
V	65	10	650	1.2
VI	38	10	380	0.7

Spectroscopic measurements of all fractions were taken and Figures 9, 10, and 11 show the various spectra. An indication of how the cytochrome containing particles were distributed in the various fractions could be determined by comparing the intensities of the peaks of the different spectra. Fractions I and II had peaks with very nearly the same intensities. This showed that after centrifugation at 60,000 x g. almost all of the cytochromes remained in solution. Fraction V had peaks which were roughly three times as intense as were those of Fraction VI. Therefore, it was evident that considerably more of the cytochrome containing particles were concentrated in Fraction V than in Fraction VI.

The diaphorase activity of all the fractions was determined by the procedure described in Materials and Methods. A 1 to 50 dilution of all fractions was made and 0.1 ml. of this solution was used in the assay. Table II lists the results that were obtained in this assay.

Table II

Diaphorase Activity of the Various Fractions

Fraction	Δ O.D. first 5 min.	Specific Activity	Total Units of Enzyme Activity
I	0.505	0.904	5050
II	0.515	1.170	3920
III	0.492	2.400	2706
IV	0.502	0.840	650
V	0.503	0.385	250
VI	0.286	0.386	143

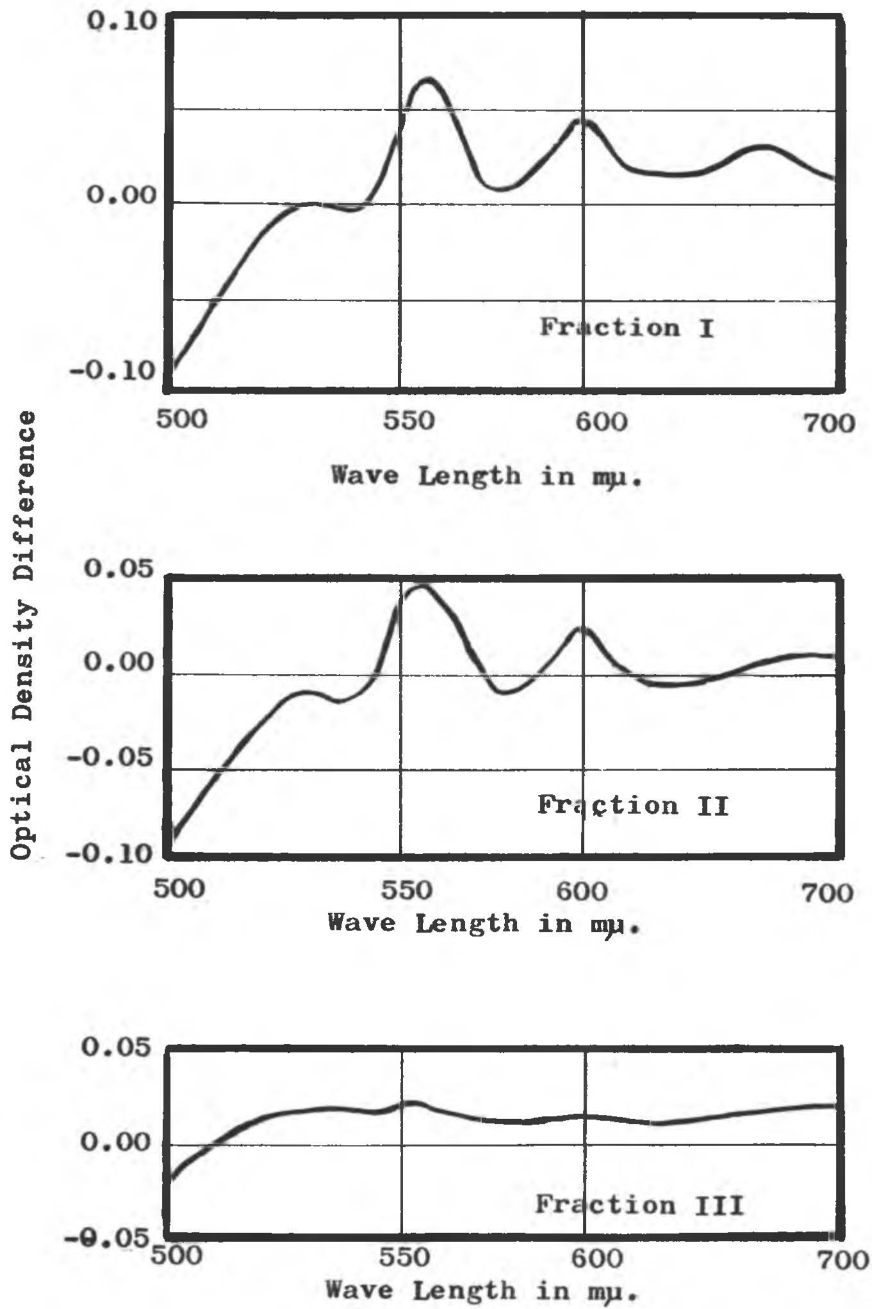

Figure 9. The difference spectra of fractions I, II, and III, obtained as in Fig. 3.

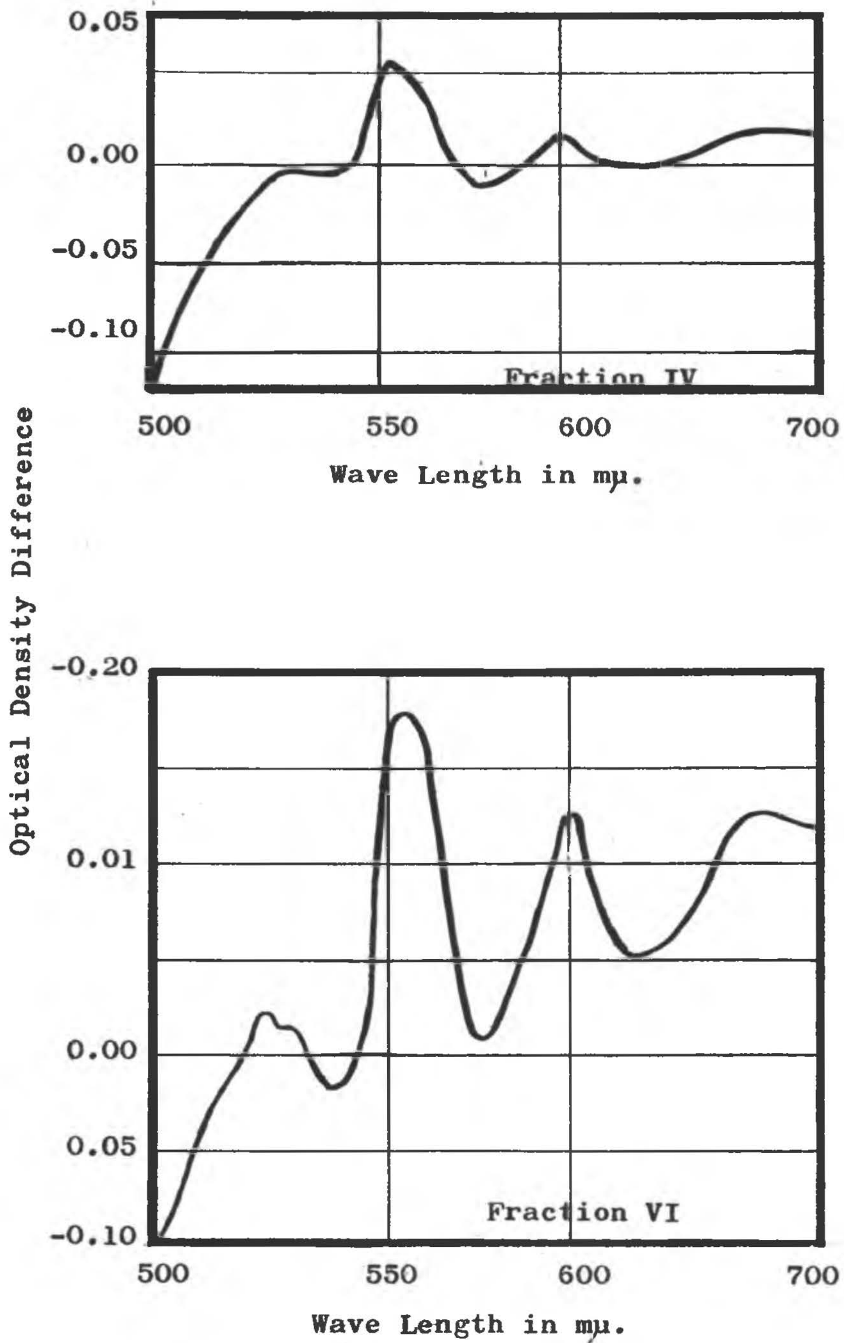

Figure 10. The difference spectra of Fractions IV and VI, obtained as in Fig. 3.

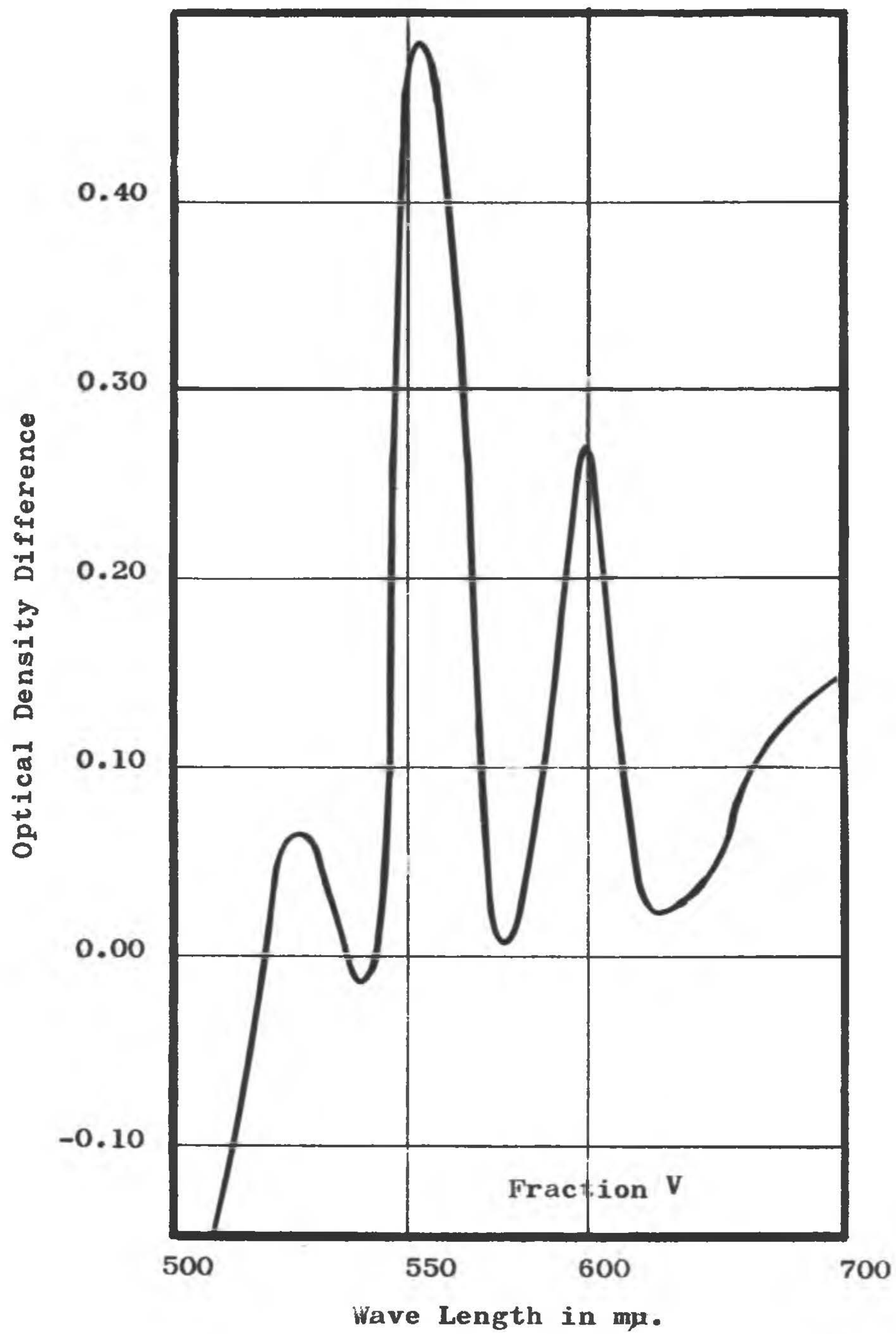

Figure 11. The difference spectrum of Fraction V, obtained as in Fig. 3.

The diaphorase activity exhibited by the different fractions tended to indicate that the enzyme responsible for this activity was a soluble entity. The fact that Fraction III had the highest specific activity showed that most of the diaphorse activity had remained in solution even after centrifugation at 144,000 x g. for three hours. The weak diaphorse activity shown by Fractions 5 and 6 was further evidence to support the conclusion that the enzyme was not particulate.

An assay for cytochrome _c_ reductase activity was conducted with a 1:10 dilution of all fractions. The results are given in Table III.

From the results in Table III there was some question as to whether the enzyme responsible for the reduction of oxidized cytochrome _c_ was a part of the cytochrome containing particle. The best specific activity was found in Fractions III and V. The cytochrome _c_ reductase activity was quite weak in all the fractions. This might be due to a specific enzyme of _B. megaterium_ acting slowly on the horse heart cytochrome _c_.

Table III

Cytochrome c Reductase Activity of the Various Fractions

Fraction	Δ O.D. first 5 min.	Specific Activity	Total Units of Enzyme Activity
I	0.090	0.031	180
II	0.094	0.043	141
III	0.075	0.063	83
IV	0.123	0.044	13
V	0.165	0.059	17
VI	0.057	0.015	6

Table IV contains the results of a DPNH oxidase activity assay of the various fractions. A 1 to 5 dilution of all the fractions was made prior to conducting the assay.

The specific activity was found to be the highest in Fractions V and VI. This observation plus the proportionately higher concentration of total units of enzyme activity in these two fractions indicated that a concentration of DPNH oxidase activity had occurred in these two particulate fractions.

In the assay system coupling the oxidation of succinate to the reduction of phenazine methosulfate, Fractions V and VI were the only ones which displayed any appreciable activity. Fraction V brought about an initial optical density change of 0.160 per minute, while the change registered with Fraction VI was 0.100. These results tended to indicate that the succinic dehydrogenase was also concentrated in the two particulate fractions. A 1 to 5 dilution of the original fractions was used in these assays.

As can be seen from Table V, all the fractions showed some cytochrome c oxidase activity. A 1 to 10 dilution of each fraction was made prior to conducting each assay.

Table IV

DPNH Oxidase Activity of the Various Fractions

Fraction	Δ O.D. first 5 min.	Specific Activity	Total Units of Enzyme Activity
I	0.063	0.011	63.0
II	0.060	0.014	45.0
III	0.028	0.012	15.4
IV	0.049	0.009	7.4
V	0.247	0.019	12.3
VI	0.194	0.025	9.7

Table V

Cytochrome *c* Oxidase Activity of the Various Fractions

Fraction	Δ O.D. first 5 min.	Specific Activity	Total Units of Enzyme Activity
I	0.040	0.014	80
II	0.032	0.015	48
III	0.008	0.007	9
IV	0.030	0.011	9
V	0.150	0.023	15
VI	0.113	0.030	11

The cytochrome c oxidase activity was concentrated in Fractions 5 and 6. Over half of the total activity was found in the 20 ml. of Fractions 5 and 6. The specific activity of Fractions 5 and 6 was over three times that of Fraction III. The cytochrome exidase was most likely located in the cytochrome containing particle.

A comparison of the cytochrome c oxidase activity of Fraction V at three different enzyme concentrations is given in Figure 12. Inhibition studies of the cytochrome oxidase were conducted using the classical inhibitors of mammalian cytochrome oxidas. The agents used were cyanide, carbon monoxide and azide. The results of these studies are given in Table VI.

The percentage inhibiton was calculated by determining the specific activity with the inhibitor present and comparing that value to the specific activity of the uninhibited system. The carbon monoxide inhibition was reversed by light. Azide only partially inhibited the oxidase activity, while cyanide caused complete inhibition.

Table VI

Inhibition of *B. megaterium* Cytochrome *c* Oxidase by Common Oxidase Inhibitors

Inhibitor	% Inhibition
Cyanide	
0.5×10^{-3} Molar	100
1.0×10^{-3} Molar	100
Azide	
0.5×10^{-3} Molar	39
1.0×10^{-3} Molar	39
Excess	89
Carbon Monoxide	
Saturated	100

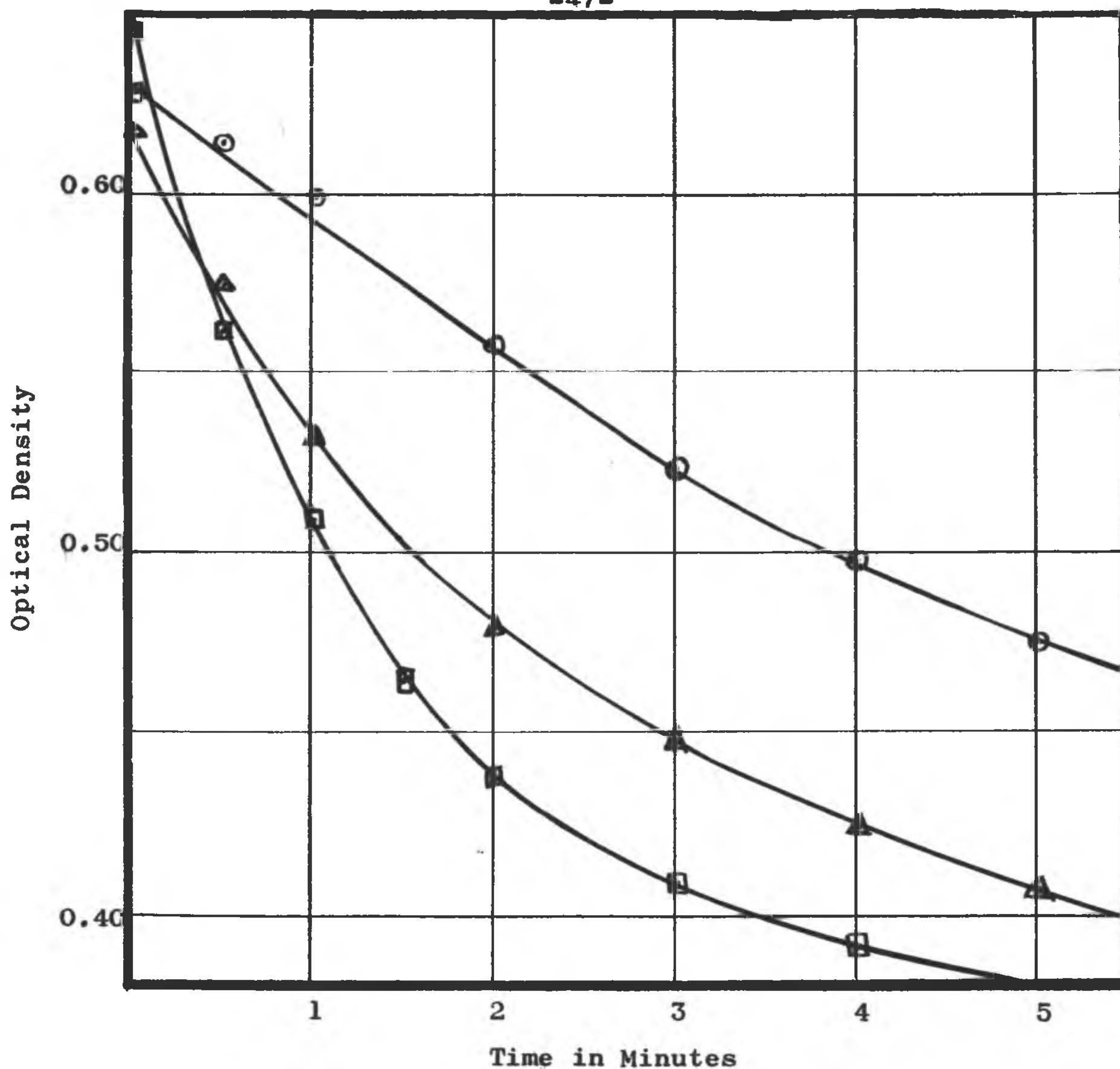

Figure 12. A comparison of the cytochrome c oxidase activity of Fraction V at three different enzyme concentrations. The circles represent a a 1:10 dilution, the triangles a 1:5 dilution, and the squares a 1:1 dilution.

DISCUSSION

It has been established by Weibull that the cytochromes of B. megaterium are contained within the cell membrane (82, 83). Since this is the case, many of the more common methods of extracting bacterial cytochromes are unable to effect a liberation of the cytochrome c of B. megaterium or B. subtilis. Warm trichloroacetic acid, a sodium hydroxide solution, pH 10, and butanol are a few of the extracting agents which were employed, but these plus several others were incapable of extracting the cytochromes. It was found that only fragmentation of the cell membrane would "solublize" the cytochromes. This could be accomplished either by sonoration or by grinding with alumina. When the cells are fragmented by either of these two methods, a particle is obtained which contains the entire cytochrome complement of the bacteria.

A spectroscopic examination of such particles confirmed the findings of other investigators (5, 6) that both B. megaterium and B. subtilis contain a cytochrome complement similar to mammalian tissue and yeast. The four banded spectrum can be sharpened and intensified by centrifuging the extract obtained from sonorated cells at 144,000 x g. for three hours. This results in the concentration of the major portion of the cytochrome bearing particles in either a pellet or in a concentrated color layer above the pellet. The peaks in the concentrated color layer are from eight to ten times more intense than those of the original sonorated extract. This concentrated fraction appears optically clear, but it does contain a large amount of non-cytochrome pigments, and for this reason it is very highly colored. With the sharpened peaks of this fraction it is possible to confirm the presence of an a type cytochrome by its absorption maximum at 600 mμ. The intensity of this peak changes from one batch of cells to the next. The intensity and shape of the peak in the region from 545 to 565 mμ. also varies greatly in the extracts obtained from different batches of cells. The greatest variation is observed in the peak at 550 mμ. The absorption at

550 mμ. is due to the presence of a cytochrome c, and in several batches of cells this has been the major alpha peak. However, in most batches of cells this peak is non-existent. These changes are difficult to explain, since apparently similar culturing conditions were employed in growing all cells. When the peak at 550 mμ. is absent from the spectrum, the amount of cytochrome c that can be extracted from these cells is insignificant. However, good yields are obtained from cells having a pronounced peak at 550 mμ. Some of the factors which might be responsible for this noticeable change in the cytochrome composition are age of the innoculum, rate of growth of the cells, age of the cells when harvested, pH, and the oxygen tension. While an attempt was made to maintain a constant pH in the growth medium, the other variables were not as carefully controlled. It was impossible to maintain exactly the same oxygen tension in each batch of cells using the apparatus described. The other factors likewise could not consistently be maintained in every growth culture. The combination of all these variables is probably responsible for the changes noted in the cytochrome content of different batches of cells.

From the many spectroscopic studies which were conducted, the presence of cytochrome of type a, b, and c in the particle has been observed. In the majority of the attempts to separate the individual cytochromes from the particle, little success was realized. However, when the particle was treated with Triton X-100 prior to digestion with the hydrolytic enzyme lipase, a fragmentation of the particle took place with the subsequent liberation of cytochrome c.

The fact that cytochrome c could be extracted from both B. megaterium and B. subtilis by means of lipase demonstrates that the cytochrome c of these two bacteria is bound in some lipid-protein combination. This binding of cytochrome c by lipid in the cell membrane explains why it is not readily extracted. Crane and Widmer (85) have been able to extract a lipid soluble cytochrome c from mammalian tissue and just recently a report (86) appeared that a phospholipase-induced release of cytochrome c from the electron transport particle of beef heart

mitochondria had been accomplished. It is interesting to note the similarities in the cytochromes isolated from the two different sources. Both are bound in some manner by a lipid-protein combination, and a lipase digestion will effect the release of cytochrome c from both sources. The partially purified cytochrome c has spectroscopic properties identical to yeast and mammalian cytochrome c. Absorption maxima are displayed at 550, 520, and 415 mμ. in the reduced form and at 408 mμ. when the cytochrome is oxidized. A sufficient amount of the partially purified cytochrome c was not obtained to characterize it further. For the same reason a final purification was not effected even though attempts were made using paper electrophoresis and ion exchange resins.

The enzymatic studies conducted with extracts of B. megaterium cells show that cytochrome c oxidase, succinic dehydrogenase, and DPNH oxidase are all particulate, and it is very probable that these enzymes are associated with the cell membrane and the cytochrome containing particle derived from the cell membrane.

The cytochrome c oxidase of B. megaterium is inhibited in a manner similar to mammalian cytochrome c oxidase by carbon monoxide, cyanide, and azide. The particulate cytochrome c oxidase shows very weak activity with horse heart cytochrome c. This may be due to a difference in specificity of the bacterial enzyme which prohibits a ready interaction with the reduced horse heart cytochrome c. It will be interesting to carry out additional enzymatic assays when enough cytochrome c is obtained from either B. megaterium or B. subtilis to make this possible.

It has been well established that the mitochondria of mammalian tissues contain particles which are able to carry on respiration in the presence of a suitable energy source (87). While the particles obtained from the fragmented cell membrane of either B. subtilis or B. megaterium have not been completely characterized, it is very probable that they have a similar function in these bacteria to that of the electron transport particle obtained from mammalian mitochondria. These two particles have much in common. For one thing both particles

contain a relatively high lipid content (81-83), Both are very highly organized, and both are capable of carrying out a similar series of enzymatic transformations. The two particles each contain the cytochrome complement of the cell. It is very likely that subsequent investigations will discover many other similarities in these two particles. In fact, the cell membrane might play the same role in the bacteria as does the mitochondria in mammalian tissue.

SUMMARY

The cytochrome content of B. subtilis and B. megaterium was shown to be associated with a particle obtained when the cell membrane was fragmented by either sonic oscillation or by grinding with alumina.

The cytochrome c from both bacteria was partially purified by digesting the fragmented cell membrane with lipase. The procedure for the partial purification of cytochrome c from B. megaterium and B. subtilis is given.

The reduced spectrum and the oxidized spectrum of the partially purified cytochrome c were determined. The absorption maxima of the reduced form were at 550, 520, and 415 mμ., while the oxidized form displayed a stromg peak at 408 mμ.

A procedure is given for concentrating the cytochrome containing particles by differential centrifugation. A comparison of the spectra obtained from several different batches of B. megaterium cells showed that the cytochrome composition varied greatly. A few batches of cells were found to have a high cytochrome c content as determined spectroscopically, while most batches had a low cytochrome c content. Variations in the content of the other cytochromes were also noted with different batches of cells. This was particularly true of the type a cytochrome.

Enzymatic studies of the various fractions showed that cytochrome c oxidase, succinic dehydrogenase and DPNH oxidase were particulate, while the diaphorase activity remained in the supernatant fluid even after being centrifuged at 144,000 x g. for three hours. Cytochrome c reductase activity was noted in both the particles and the supernatant fluid.

Inhibition studies on the cytochrome c oxidase were conducted. Cyanide and carbon monoxide brought about a complete inhibition, while azide was only partially effective as an inhibitor. The carbon monoxide inhibition was light reversible.

A discussion is presented comparing mammalian cytochrome *c* with the bacterial cytochrome *c* partially purified from *B. megaterium* and *B. subtilis*. A comparison was also made between the electron transport particle of beef heart mitochondria and the cytochrome containing fragmented cell membrane.

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THE CYTOCHROME COMPOSITION OF *BACILLUS SUBTILIS* AND *BACILLUS MEGATERIUM*

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ABSTRACT

A spectroscopic and enzymatic investigation of the cytochromes of B. megaterium and B. subtilis was conducted. Attempts were made to isolate and characterize the individual cytochromes as well as to determine the locale of such activities as cytochrome c oxidase, DPNH oxidase, succinic dehydrogenase, cytochrome c reductase and diaphorse.

Only two methods proved effective in liberating the cytochromes; disruption of the cells with sonic osillations and grinding the cells with alumina. These procedures caused a fragmentation of the cell membrane. It was found that the entire cytochrome complement of these two bacteria was bound intimately to particles which most likely arise from the cell membrane.

The spectrum of these cytochrome containing particles displayed the typical four banded spectrum found in mammalian tissues and yeast. From the various studies made, the presence of cytochromes of the a, b, and c type was confirmed. A concentration of the cytochrome bearing particles could be brought about by either an ammonium sulfate fractionation or by centrifugation at 144,000 x g. for three hours. When the particles were centrifuged, a concentration of these particles in an intense color layer at the bottom of the centrifuge tube resulted. The spectrum of this concentrate showed peaks which were several times more intense than were those shown by the original sonic extract. With these sharpened peaks it was easier to note changes in cytochrome composition of different batches of cells. A few batches of cells contained a high content of cytochrome c, while most batches of cells had only a low content of this particular cytochrome. The content of the a type cytochrome also varied markedly in the different batches of cells. The content of cytochrome b was more resistant to change, but even the amount of this cytochrome showed some variation.

A procedure for obtaining a partially purified cytochrome c

from the cytochrome concentrate is presented. Extraction was brought about by pre-treating the fraction with Triton X-100 before performing a digestion with the hydrolytic enzyme lipase. This treatment liberates a soluble cytochrome c which can be precipitated from a concentrated ammonium sulfate solution with trichloroacetic acid in the usual manner.

The absorption maxima of the reduced spectrum of the partially purified cytochrome were at 550, 520, and 415 mμ. The Soret peak shifted to 408 mμ. when the pigment was oxidized.

A short discussion of the similarities between this bacterial cytochrome c and a lipid soluble cytochrome c obtained from mammalian tissues is included.

The enzymatic studies showed that cytochrome c oxidase, DPNH oxidase, and succinic dehydrogenase are associated with the particles described above, while the diaphorase and cytochrome c reductase were found in the more soluble fractions.

Inhibition studies with cytochrome c oxidase, using cyanide, carbon monoxide and azide revealed a similar inhibiton pattern to that shown by mammalian cytochrome oxidase.