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THE CYTOCHROME SYSTEM OF *BACILLUS MEGATERIUM* KM. THE PRESENCE AND SOME PROPERTIES OF TWO CO-BINDING CYTOCHROMES

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SUMMARY

1. Difference spectra of *Bacillus megaterium* KM membrane preparations indicate the presence of two pigments which bind CO and which exhibit the spectral characteristics of cytochromes a_3 and *o*. Relative amounts of the pigments vary with growth stage of the organism, but both are present at all stages which have been investigated. The pigments are believed to be metabolically active because they are completely reducible by substrate (NADH) and are reoxidizable in the presence of air. CO difference spectra of whole cell suspensions are in agreement with spectra of the isolated membrane fragments. In particulate preparations and in whole cells, CO difference spectra suggest that the a_3 component binds CO much more readily than the *o* component; this behavior offers a possible explanation for the fact that cytochrome *o* has been detected in only a few other microorganisms, since CO binding is by definition the property used to identify this cytochrome.

2. A separation of the two CO-binding pigments is obtained by incubation of membrane preparations with pancreatic lipase. This treatment selectively removes the *o* pigment from the membrane, leaving the a_3 component associated with an enzymatically active particulate fraction.

INTRODUCTION

The complex systems of membrane-bound enzymes that carry out electron transport offer limited possibilities for study of the role of individual components; in general, it is not possible to modify the relative amounts of pigments in isolated electron-transport particles experimentally without destroying respiratory activity. Bacteria, however, show considerable variation in the type and quantities of respiratory pigments that they contain. Not only are there significant differences between various species but also in the same microorganism cultured under different conditions[1]. WEIBULL[2,3] was able to isolate a membrane fraction from bacteria and to show that the respiratory chain system was localized in that fraction. Using his methods on cells subjected to different growth conditions before harvest, one can obtain membrane preparations from a single microorganism that differ in pigment content but which all catalyze O_2 uptake by substrate. Indirectly, the role of some of the individual members of the electron-transport chain can be assessed.

Only a few studies of the respiratory pigments of *Bacillus megaterium* have been published. Early spectroscopic studies of whole cell suspensions gave reduced visible α bands which indicated the presence of an *a* type cytochrome at 603 mμ and a *b* type cytochrome at 560 mμ (ref. 4). WEIBULL's absorption spectra of the isolated membrane fraction of the KM strain similarly indicated two reduced α peaks (at 600 and 558 mμ) *plus* a large Soret band[3]. WEIBULL AND BERGSTROM[5] also presented absorption spectra for the M strain of the organism which differed from the KM strain and possessed a third visible spectral component at 552 mμ, indicating the presence of a *c* type pigment in addition to the *a* and *b* types. VERNON AND MANGUM[6], using a strain of *B. megaterium* not identified as either the KM or the M strain, obtained results very similar to those of WEIBULL AND BERGSTROM[5]; reduced *minus* oxidized difference spectra gave evidence for *a*, *b*, and *c* type cytochromes.

The present spectral study confirms the presence of *a* and *b* type cytochromes in the KM strain and extends the previous work by presenting quantitative variations that occur at different growth times and by studying CO binding of the spectral components. CO studies indicate two different CO-binding pigments, one an a_3 type and one an *o* type cytochrome. The *o* type component can be selectively removed from the membrane fragments leaving a functional electron-transport chain with CO binding characteristic of a single a_3 type pigment.

METHODS

Cultures of *B. megaterium*, strain KM, originally obtained from Dr. R. STORCK, were grown in 2 % Bacto-peptone broth at 30° on a New Brunswick rotary shaker. Cells were harvested at the times indicated for each experiment by centrifugation at 7000 $\times$ *g* for 5 min and washed once with distilled water. For whole cell experiments, the washed cells were resuspended in sterile 2 % Bacto-peptone, and spectra were run immediately on the homogenized cell suspension.

To prepare particle suspensions, each gram wet weight of washed whole cell residue was resuspended in 20 ml of 0.5 M sucrose–0.01 M phosphate (pH 7.0). Sigma Grade I crystalline lysozyme (EC 3.2.1.17) dissolved in the same concentration of buffered sucrose solution was added to the cell suspension to give a final concn. of 0.2 mg lysozyme/ml. Incubation at 25° was continued until conversion to single protoplasts was complete as determined with a phase contrast microscope. Protoplasts were separated from cell wall material by centrifugation at 15000 $\times$ *g* for 30 min in a Servall refrigerated centrifuge. In contrast to WEIBULL's report[7] that the presence of Mg^{2+} in the suspending medium was required to prevent lysis of protoplasts even in hypertonic media, it was found that the protoplasts obtained were completely stable to centrifugation without the addition of Mg^{2+}.

Osmotic lysis of the protoplast residue was effected by adding 0.01 M phosphate buffer (pH 7.0) containing Worthington crystalline deoxyribonuclease (EC 3.1.4.5); 10 ml of the phosphate buffer, containing 0.008 mg deoxyribonuclease, were used for the protoplast residue obtained from each gram wet weight of whole cells. The mixture was stirred at room temperature for 10 min, then centrifuged at 33000 $\times$ *g* for 1 h in a Servall refrigerated centrifuge to collect the membrane fragments. The deeply yellow colored residue was taken up in 0.01 M phosphate buffer (pH 7.0) and homogenized; 2 ml of the buffer were used for each gram wet weight of whole cells.

Membrane preparations were routinely made up to this concentration and if not used immediately were frozen at this step.

All difference spectra were run in a Model 14 Cary recording spectrophotometer supplied with a strong light source and with a 0–0.1 absorbance slide wire. For most membrane preparations, a 1:3 dilution of the preparation described above gave suitable reduced *minus* oxidized difference spectra. The suspensions of membrane fragments were reduced by adding either a few grains of dithionite or 4–8 μmoles of NADH (Sigma). CO (Matheson) was bubbled through the reduced membrane suspensions for the times indicated in the RESULTS section. Protein determinations were made using a modified biuret method[5,8].

Assays of respiratory activity were made with a Clark oxygen electrode. Volume of the test solution was 3.0 ml; temperature was 25°. Four μmoles of NADH (Sigma), or 40 μmoles of D-L malate were used as substrate.

To prepare a lipase suspension for incubation experiments, 3.5 g of crude pancreatic lipase (EC 3.1.1.3) from General Biochemicals, Inc., were stirred with 210 ml 10% NaCl for approx. 7 h at room temperature. A large amount of insoluble material was removed by centrifugation at 7000 $\times$ *g* in a refrigerated Servall centrifuge for 10 min, and recentrifugation of the supernatant from the first spin at the same speed and time. The resulting yellow solution was filtered and stored in a freezer until used.

Lipase incubations were carried out as follows: Two 5-ml aliquots of a membrane particle preparation were centrifuged at 33000 $\times$ *g* for 1 h. The residue from one aliquot was taken up in 6.0 ml of the lipase extract described above. The residue from the second aliquot was taken up in 6.0 ml of 10% NaCl. These two mixtures *plus* a third sample of 6.0 ml of the lipase extract were incubated at 37° for 7 h. At the end of this time, the incubated mixtures were centrifuged at 33000 $\times$ *g* for 1 h, and the supernatants decanted off. Each of the supernatant and residue fractions was made up to 6.0 ml with 10% NaCl and 0.5 ml of 0.5 M phosphate (pH 7.0) was added. Difference spectra were run on each of the samples without further dilution.

RESULTS

Reduced minus oxidized difference spectra; effect of growth time

Dithionite-reduced *minus* oxidized difference spectra were obtained for membrane preparations from *B. megaterium* cells harvested at 6, 12, 24, and 48 h of growth. Each of 17 preparations tested gave difference spectra consisting of visible α peaks at 602 and 557 mμ and Soret peaks at 443 and 427 mμ. Relative heights of these peaks varied considerably in different preparations, but particles from cells harvested at the earlier times showed prominent 602 and 443 mμ peaks characteristic of *a* type cytochromes and fractions from cells grown for longer times showed prominent 557 and 427 peaks characteristic of *b* type cytochromes. Spectra of particles harvested at three different time intervals are pictured in Fig. 1. It appears that membrane fractions from cells late in stationary phase (B and C in Fig. 1) contain a much higher ratio of *b*/*a* cytochrome than do similar fractions from cells grown for shorter times.

Comparison of NADH reduction and dithionite reduction

The clearly separated α peaks of reduced *minus* oxidized difference spectra were used to compare the extent of reduction of the *a* and *b* components by substrate

(NADH) and by dithionite. It was determined that isosbestic points existed at 458, 575, and 630 mμ and the two latter wavelengths were used as reference points for calculations of the two visible peaks. In Table I the results of these calculations are presented for 6 preparations, some of which had been frozen, some lyophilized, and some measured without either treatment. Very little difference between the two

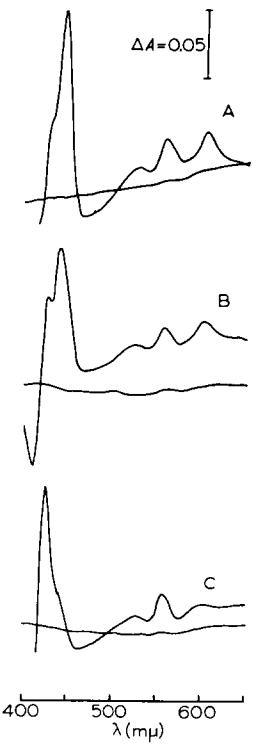

Fig. 1. Reduced *minus* oxidized difference spectra of membrane fragments prepared from *B. megaterium* cells harvested at different growth stages: A, 12 h; B, 24 h; C, 48 h. Samples were reduced with a few grains of solid $Na_2S_2O_4$ and read against untreated preparations. The base line included with each spectrum was obtained by running two untreated suspensions. Samples A and B contained 2 mg protein/ml; Sample C contained 5 mg protein/ml.

TABLE I

COMPARISON OF NADH AND DITHIONITE REDUCTION OF MEMBRANE FRAGMENTS OF *B. megaterium*

Reduced *minus* oxidized difference spectra; heights of α peaks calculated as described in text.

Treatment of membrane fragments	*Time of growth before harvest (h)*	$10 \times \Delta A_{557-575\ m\mu}$		$10 \times \Delta A_{602-630\ m\mu}$	
		Dithionite	*NADH*	*Dithionite*	*NADH*
Untreated	48	0.16	0.16	0.16	0.11
Untreated	48	0.29	0.28	0.10	0.12
Frozen	12	0.17	0.17	0.22	0.22
Frozen	24	0.10	0.10	0.17	0.14
Frozen	48	0.19	0.20	0.04	0.09
Lyophilized	26.5	0.22	0.22	0.12	0.13

reductants is seen; from this it is concluded that the cytochrome system is completely reducible by substrate and stable to the storage procedures used.

CO-reduced minus reduced difference spectra of membrane fragments

CO difference spectra were run to determine the contribution of cytochrome a_3 to the total reduced peak at 443 mμ. A suspension of membrane particles reduced by dithionite and bubbled briefly (30 sec) with CO read against a second sample of reduced particles gave a steep symmetrical Soret trough at 444–445 mμ and a sharp peak at about 425 mμ adjacent to it; visible spectra showed only a very small peak at 590 mμ. Table II gives calculations of the extent of change in absorbance of the Soret peak due to CO binding. Values are presented for 6 membrane preparations obtained from cells grown for 12 h. The ratio of the CO trough to the reduced peak at 443 mμ is assumed to be a measure of the $a_3/(a+a_3)$ content of the preparation. These ratios vary from 0.25 to 0.47. The a_3 component therefore accounts for less than half of the absorption of the 443-mμ peak.

TABLE II

CONTRIBUTION OF CO-BINDING COMPONENT TO THE 443-mμ SORET PEAK OF REDUCED *minus* OXIDIZED DIFFERENCE SPECTRA OF *B. megaterium* MEMBRANE FRAGMENTS

In all experiments, bacteria grown for 12 h before harvest. Reduction carried out by addition of a few grains of solid dithionite to particle preparations. CO bubbled for 30 sec through the dithionite-reduced suspensions.

CO trough $10 \times \Delta A_{443-458\ m\mu}$	*Soret peak* $10 \times \Delta A_{443-458\ m\mu}$	*Ratio*
0.83	2.05	0.40
1.95	4.14	0.47
1.63	4.39	0.37
2.34	5.74	0.41
0.90	2.61	0.34
0.60	2.34	0.25

On longer bubbling with CO, particle suspensions gave evidence for a second CO-binding pigment. Its spectrum was superimposed on that of the a_3, and the overall spectral change was a result of the relative amounts of the two pigments and of the length of time of CO bubbling. In preparations having a very high proportion of the second pigment, the a_3 contribution was minimal, and the Soret CO trough was seen at around 430 mμ; its peak was at 410–415 mμ. In the visible region, the main feature of the spectrum was a trough at 563–565 mμ. Compared to the a_3–CO compound, the CO complex of the second pigment showed greater absorption in the visible region. Because its spectral properties resemble those of cytochrome *o* reported as a terminal oxidase in several organisms, the second pigment is tentatively considered to be *B. megaterium* cytochrome *o*.

The observation that one 30-sec bubbling of a suspension of membrane fragments was sufficient to give maximum CO binding of the a_3 but was insufficient to demonstrate significant CO binding of the second pigment led to attempts to determine conditions which would permit maximum CO binding of both. Successive 30-sec bubblings with CO caused spectral changes due to a stepwise increase in the amount of the cytochrome *o*–CO complex with no observable change in the initial 445-mμ

trough caused by the formation of a_3–CO. When the *o* concentration of a particular sample was low, it appeared to be saturated by a few brief CO bubblings. However, when the *o* content was very high, it appeared that even a total of 15 min of bubbling with CO would not saturate the system. Presumably either greater than 1 atm of CO and/or possibly a very low level of O_2 is required for maximum CO binding by cytochrome *o*. For this reason, no quantitative values for the cytochrome *o* content of *B. megaterium* can be obtained from difference spectra run at atmospheric pressures of CO. Even the values for cytochrome a_3 that can be obtained from CO difference spectra are valid only when the cytochrome a_3 content is high relative to the cytochrome *o*, because in the absence of significant amounts of cytochrome a_3 a small amount of CO is bound by the cytochrome *o* component during a single 30-sec bubbling.

CO difference spectra of whole cells

To answer the question of whether cytochrome *o* was a natural constituent of *B. megaterium* cells or whether it was an artifact due to the procedures used in preparation of the membrane fractions, CO difference spectra were also run on suspensions of whole cells harvested at different growth stages. These experiments made use of the fact that this strain of *B. megaterium* has a very high rate of endogenous respiration and a very large reserve of endogenous substrate at all but very late growth stages. This meant that cells resuspended in fresh sterile peptone after harvest so that even late growth stage organisms would have an excess of substrate available remained completely anaerobic even during extensive bubbling. Since CO binding depends on the presence of the reduced form of the respiratory pigments, the CO difference spectra of whole cells must indicate pigments which bind CO when they are reduced *via* substrate.

In Fig. 2, CO difference spectra are shown for whole cells harvested when absorbances of the cultures measured at 550 mμ, had reached 0.110, 0.200, 0.340, 0.345, 0.390 and 0.380*. One experiment was used for the first three points, another for the last three, with one point from each experiment taken at approximately the same growth stage for comparison between the two runs. For each time point, two spectra are shown, one after a single 30-sec bubbling and one after extensive CO bubbling. For one time point (absorbance = 0.345) some intermediate spectra are also shown to indicate the specific increase in only one component with successive CO treatments. In the oldest cells, the proportion of cytochrome *o* to cytochrome a_3 is so high that the 443–445-mμ trough is asymmetric or double.

The spectra obtained show all the features of spectra from suspensions of membrane fragments. All cell suspensions indicate the presence of two CO-binding pigments; earliest cells have the highest relative concentration of cytochrome a_3 and the latest cells have the highest proportion of cytochromes *o* to a_3. A single 30-sec bubbling gives maximum binding of CO by cytochrome a_3 but longer exposure is required to give CO binding of cytochrome *o*. Since it was possible to demonstrate spectrally the same CO-binding pattern in whole cells reduced only by substrate as was seen in isolated membrane preparations reduced with dithionite, cytochrome *o* with its low affinity for CO is believed to be a natural constituent of the organism.

* Turbidity measurements of growing cultures ($A_{550\ m\mu}$) were made with a Coleman Junior spectrophotometer.

Lipase treatment of isolated membrane fragments

The two CO-binding pigments must also differ in their attachment to the membrane because it is possible to remove one of them without disturbing the other. A suspension of membrane fragments which had been incubated with pancreatic lipase and then collected by centrifugation gave dithionite-reduced *minus* oxidized difference spectra (Fig. 3) and CO difference spectra (Fig. 4) of the residue and supernatant fractions which indicate that the *o* pigment is no longer sedimentable after the lipase treatment but that the a_3 pigment remains associated with the residue.

Fig. 3A gives the spectra of the treated membranes *plus* those of the residues from control incubations. The membranes incubated with only NaCl indicate the

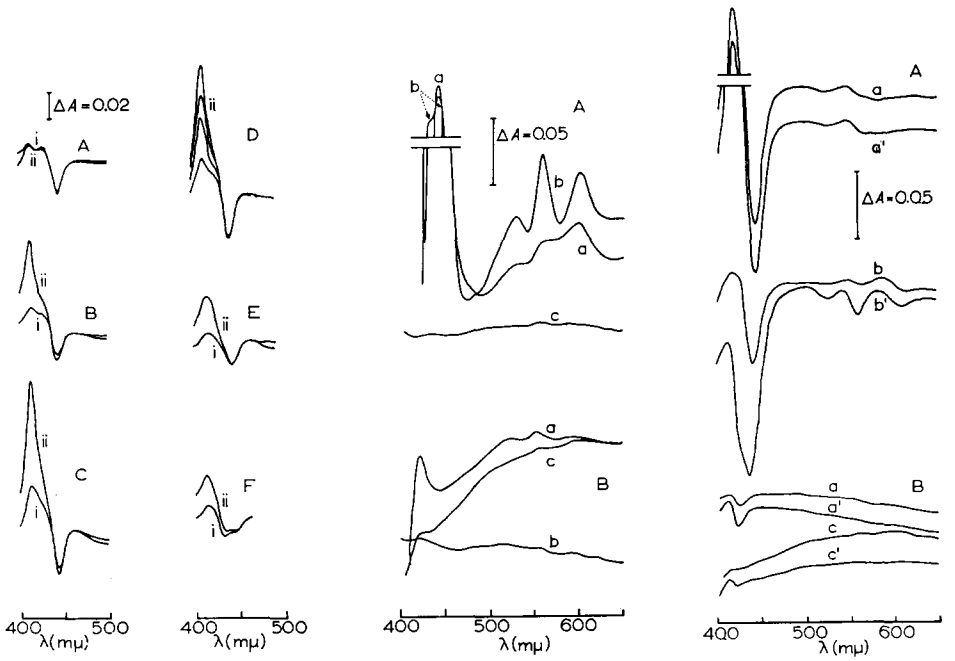

Fig. 2. CO difference spectra of whole cell suspensions of *B. megaterium* harvested at different growth stages. $A_{550\ m\mu}$ of growing culture and time of harvest for each sample are: A, 0.110, 4 h; B, 0.200, 4.5 h; C, 0.340, 7.5 h; D, 0.345, 8.5 h; E, 0.390, 24 h; F, 0.380, 48 h. For each time point, i represents the difference spectrum obtained after a single 30-sec bubbling of sample with CO and ii represents the curve obtained after extensive CO bubbling. Additional curves in D are those obtained after bubbling with CO for intermediate time periods. Pigments are reduced with endogenous substrate; see note in text.

Fig. 3. Effect of pancreatic lipase on *B. megaterium* membrane fragments. Dithionite-reduced *minus* oxidized difference spectra of residue (A) and supernatant (B) fractions from incubation of membrane preparations with crude pancreatic lipase. Incubation mixtures: (a) membranes *plus* pancreatic lipase; (b) membranes *plus* 10% NaCl; (c) pancreatic lipase. Incubation was carried out at 37° for 7.0 h, followed by centrifugation at 33000 × *g* for 1 h.

Fig. 4. Effect of crude pancreatic lipase on isolated membrane fragments of *B. megaterium*. CO difference spectra of residue (A) and supernatant (B) fractions obtained from incubation of membranes with lipase as in Fig. 3: (a) membranes *plus* lipase; (b) membranes *plus* 10% NaCl; (c) pancreatic lipase. Curves labeled a, b, c are those obtained after a single 30-sec bubbling with CO; Curves a′, b′, c′ are those obtained after bubbling with CO for 5 min.

presence of significant amounts of *a* and *b* type cytochromes; in contrast, the lipase-treated membranes contain predominantly the *a* type, and almost all of the *b* type is missing. The effect of the lipase treatment is particularly evident in the visible region. In the control residue (Fig. 3A), the 557-mμ peak is the highest visible peak observed, but in the treated residue, it is only a minor component. In the Soret region, the control residue has a large double peak; in the lipase-treated residue, only the single 443-mμ peak can be seen. There is no evidence for even a shoulder at the lower wavelength of the other component.

A dithionite-reduced *minus* oxidized difference spectrum of the supernatant fraction from the lipase *plus* membranes incubation indicates a single Soret peak at about 425 mμ and a small visible peak at about 555 mμ. The material in this fraction in contrast to all other preparations from *B. megaterium*, was not completely reduced immediately upon adding dithionite. Only about half of the maximum change in absorbance was observed initially; approx. 45 min were required for the maximum change. All spectra in Fig. 3 were obtained by waiting until the maximum absorbance changes had occurred. The small amount of material exhibiting a reduced peak at 425 mμ present in the lipase control supernatant did not increase with time after adding reductant.

CO spectra of residues from lipase incubation and NaCl control incubation are compared in Fig. 4A. The NaCl control incubation, which released no reducible material and no CO-binding material into the supernatant (Figs. 3B and 4B) shows a spectrum typical of an untreated membrane preparation, with two CO-binding pigments, cytochromes a_3 and *o*, the latter increasing specifically on longer exposure to CO to give an asymmetric trough and to lower the maximum wavelength of the peak adjacent to it. The lipase-treated residue appears to contain only one CO-binding pigment with the characteristics of cytochrome a_3. There is no increase in CO binding when bubbling is extended; the trough remains the same with a minimum at 443 mμ and the adjacent peak remains the same height and is not shifted to a lower maximum wavelength. It appears that the lipase treatment has specifically removed cytochrome *o* from the membrane and left cytochrome a_3 firmly attached to it. The NaCl incubation has removed nothing from the membrane.

CO spectra of the supernatant fractions presented in Fig. 4B indicate that the 425-mμ-reduced material found in the supernatant after lipase treatment does bind CO and that the CO binding is incomplete after a 30-sec bubbling. The small amount of 425-mμ-reduced material in the lipase control supernatant also binds CO but this is changed only slightly by extending the CO bubbling. Attempts to purify the lipase extract gave material with no reduced spectrum in the visible region and no CO binding, but much less activity toward the *B. megaterium* membranes.

Aliquots of the lipase-treated residue, the NaCl-treated residue, and the initial membrane preparation were tested for NADH oxidase activity with an oxygen electrode. All three were enzymatically active, the lipase-treated residue having activity equal to the untreated membrane fragments and the NaCl control with about 2/3 the activity of the others. The full activity of the lipase-treated residue indicates that the pigments that remain membrane bound after lipase treatment are functional; removal of the second CO-binding pigment has not lowered activity. No O_2 uptake was found for the material removed from the chain using NADH or malate, the two most active substrates for the isolated membrane fraction of this organism.

DISCUSSION

Reduced *minus* oxidized difference spectra of membrane fragments from *B. megaterium*, strain KM, give evidence for the presence of only *a* and *b* type cytochromes. However, suspensions of reduced membrane particles, exposed to CO, indicate that a portion of both components combine with and give spectral changes that are characteristic of cytochrome *o* and cytochrome a_3 in other microorganisms. SMITH[9], using the method of CHANCE[10], compared the CO difference spectra of *Micrococcus pyogenes*, var. albus*, which contains cytochrome *o* as its only CO-binding pigment, with spectra of *B. subtilis*, which contains cytochrome a_3 as the only CO-binding respiratory pigment. The spectra obtained from *B. megaterium*, strain KM, exhibit the properties of both; they are the spectra that would be obtained from preparations which contain significant amounts of both pigments.

Cytochrome *o*, which is identified as a CO-binding pigment with a reduced Soret absorption band in the region of the *b* type cytochromes, apparently functions as the only or the main terminal oxidase in several organisms. In addition to *Micrococcus pyogenes*, var. albus, *Acetobacter suboxydans* was found by photochemical action spectra to contain cytochrome *o* as the only oxidase[11]. In particles from aerobic dark-grown *Rhodospirillum rubrum*, TANIGUCHI AND KAMEN[12] found that there is no *a* type cytochrome present and that the CO difference spectra and photochemical action spectra gave evidence for cytochrome *o* as the only functional oxidase. TABER AND MORRISON[13] studied a particle preparation of *Staphylococcus aureus*, which does contain an *a* type component but no cytochrome a_3, and on the basis of CO difference spectra and photochemical action spectra, conclude that cytochrome *o* is the only functional oxidase in this organism also. Using *Staphylococcus epidermidis*, JACOBS AND CONTI[14] found evidence for cytochrome *o* as the principal oxidase on the basis of CO difference spectra of cells grown aerobically or grown anaerobically and supplemented with hemin.

Several organisms have been shown by CASTOR AND CHANCE[11] to contain functional cytochrome *o plus* cytochrome a_1 and/or a_2. However, the present finding of significant amounts of both cytochromes a_3 and *o* in *B. megaterium* is unusual. SATO[15] stated that a_3 and *o* were present together in whole cells of *Micrococcus denitrificans*, but that the concentration of *o* was much less than that of a_3. No spectra were presented so that a comparison with the present work is not possible. DWORKIN AND NIEDERPRUEM[16] studied cholate-treated particles from vegetative cells of *Micrococcus xanthus* and obtained CO difference spectra that could indicate both *o* and a_3. The treated particles were non-specifically reduced by dithionite and the authors comment that the results may be affected by the preparative procedures used. It is possible that the presence of cytochrome *o* in other systems has been overlooked if the low affinity for CO that has been observed in *B. megaterium* is true for the cytochrome *o* of other organisms as well. Differences in the reactions of cytochromes *o* and a_3 with CO have been suggested by the differences in photodissociation of the two cytochrome–CO complexes observed by CHANCE[17].

Certainly the suspension of crude pancreatic lipase used here contained several enzymatic activities so that the nature of the effective digestive process is unknown.

* Nomenclature used for microorganisms is in each case that given by the author of the work quoted.

However, similar preparations of pancreatic lipase have been shown to react with intact bacterial membranes in such a way that the permeability properties are drastically changed. SPIEGELMAN, ARONSON AND FITZ-JAMES[18] tested the enzyme for its effect in lysing protoplasts of *B. megaterium*, and the lysis has been confirmed in this laboratory on protoplasts of the strain of *B. megaterium* used for membrane preparations. Very little is known about the chemical changes brought about in the membrane as a result of the enzymatic treatment, but its significant feature is that it affects individual respiratory pigments. VERNON AND MANGUM[6] report that a 12-h lipase treatment of membranes isolated from their strain of *B. megaterium* appeared to remove a single component of the respiratory system, a *c* type cytochrome. Both *a* and *b* type cytochromes were present but were not removed by the treatment. In the present work, a shorter exposure to lipase of an electron-transport chain from *B. megaterium*, which appears to contain no *c* type cytochrome, specifically removes material with a reduced peak at the wavelength of *b* type cytochromes but with the CO-binding properties of *o* type cytochromes. The data suggest that though all the pigments present are reducible by substrate, the nature of the linkages of individual pigments to the membrane must differ to account for the specific removal of only one CO-binding pigment by lipase treatment.

KEILIN[19] has stated that no case has been recorded of a ferrohaemoprotein that combines with CO but does not react with O_2; however, without photochemical action spectra, it cannot be assumed that both the CO-binding pigments observed in this study serve as terminal oxidases in intact *B. megaterium* cells. Indirectly, one can test for respiratory activity of the *o* component by measuring O_2 uptake catalyzed by a lipase-treated membrane preparation on the addition of substrate. The present results suggest that since activity is not impaired in the treated particle preparation, cytochrome *o* is not essential for oxidation of NADH in this organism. The data are in agreement with CASTOR AND CHANCE's results[11] using stationary phase *Escherichia coli*; there both cytochromes *o* and a_2 are present together, and apparently either pigment can catalyze most of the respiration.

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