oxidizing agents and their suggested inhibition[3] of the BAL-sensitive factor remains obscure.

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A CYTOCHROME OF THE *b* GROUP FROM *MICROCOCCUS LYSODEIKTICUS*

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SUMMARY

A method has been devised for separating the cytochrome *b* component of *M. lysodeikticus* from the other cytochromes. The absorption maxima of the reduced component in whole cells, lysates and particulate preparations were at about 560–561 mμ, 525–530 mμ and 429–430 mμ.

The separated cytochrome *b* component was auto-oxidizable, even in the presence of cyanide ($5.0 \cdot 10^{-2}$ *M*). Observations suggest that its potential is lower than that of the bacterial cytochrome *c*.

The cytochrome *b* component could be solubilized by treatment of the particulate preparation with 5 % w/v "Cetrimide" solution, but precipitation of the cytochrome from the solution with ammonium sulphate produced small shifts in the absorption maxima.

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INTRODUCTION

In an investigation of respiratory inhibition produced by certain quinoline-N-oxides[1,2], it was found that these materials prevented the oxidation of reduced cytochrome *b* components in both heart-muscle and bacterial preparations. It seemed probable that in heart muscle, and in those bacterial preparations which contained cytochrome *c*, the block in the electron-transport system was at a site between cytochrome *b* and cytochrome *c*.

The quinoline-N-oxides are of particular microbiological interest, because they antagonize the inhibitory action of streptomycin against some micro-organisms[3]. As part of a more complete investigation of their mode of action, it was necessary to prepare the cytochrome *b* component of a susceptible bacterial species free from other cytochromes, and the present paper describes a method which has been devised for this purpose. The organism chosen, *Micrococcus lysodeikticus*, is easy to handle in bulk, since it lyses readily on treatment with lysozyme.

A preliminary account of some of this work has already been published[4,5].

MATERIALS AND METHODS

Organisms

For small-scale experiments, *M. lysodeikticus* (N.C.T.C. 2665) was grown in 50-ml vols. of meat digest broth in 250-ml Erlenmeyer flasks at 35°; the flasks were shaken at 120 oscillations/min, amplitude 20 mm, to ensure adequate oxygenation. Organisms were harvested by centrifugation after 20 h incubation, washed twice with distilled water and suspended for lysis with lysozyme in a solution made by mixing 4 vols. 0.04 *M* phosphate buffer, pH 7.0, and 1 vol. 0.9 % NaCl.

A "pale", relatively less pigmented variant of the yellow N.C.T.C. 2665 strain, was selected from culture plates, and was used in some experiments.

For a large-scale experiment, the bulk culture of 1.5 kg *M. lysodeikticus* (N.C.T.C. 2665) was kindly arranged by Dr. D. W. HENDERSON, of the Microbiological Research Station, Porton, England.

Dry weights were determined by heating measured volumes of materials at 110° to constant weight, and optical density dry weight relationships of whole-cell suspensions were measured with a Hilger Spekker absorptiometer.

Spectroscopy and spectrophotometry

Direct visual spectroscopic observations were made by means of a Beck-Hartree microspectroscope and a 150 c.p. "Pointolite" at room temperature and after cooling the specimens in liquid oxygen. Spectrophotometric examination of the materials was carried out in a Hilger "Uvispek" absorptiometer.

The cytochromes were reduced by addition of solid $Na_2S_2O_4$ (approx. 1 mg to each ml of solution or suspension). Insoluble materials were suspended in glycerol (final concn., 50 %) to reduce light scattering.

Reagents

Lysozyme was prepared from egg white by the direct precipitation method of FEVOLD AND ALDERTON[6].

Ribonuclease and mammalian cytochrome *c* were obtained from L. Light and Co.

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Ltd., Colnbrook, England; deoxyribonuclease ("Derinase") was kindly supplied by Bioindustria, Novi Ligure, Italy, and Takadiastase by Parke, Davis and Co. Ltd., Hounslow, England.

The 2-heptyl-4-hydroxyquinoline-N-oxide was obtained from Dr. J. W. CORNFORTH.

All phosphate buffers were prepared with KH_2PO_4 and NaOH, and acetate buffers with sodium acetate and acetic acid.

EXPERIMENTAL

Separation of the cytochrome b *component from other cytochromes*

The method is best illustrated by describing a typical experiment; the stages are indicated in Fig. 1.

A freshly collected batch of *M. lysodeikticus* (6.0 g dry wt.) was suspended in the saline buffer solution (100 ml), and 100 mg egg-white lysozyme and 10 mg ribonuclease were added. The suspension was shaken for 30 min at 35°, by which time lysis was complete. Preliminary experiments had shown that the addition of ribonuclease reduced the viscosity of the material after lysis, and made it easier to handle. Addition of deoxyribonuclease rendered the material completely fluid, but it was found that the separation of the cytochrome *b* component from cytochrome *c* became more difficult under these conditions.

The lysate was made up to 300 ml with distilled water, and ammonium sulphate (120 g) was added with stirring. The suspension was centrifuged at 20,000 × *g* for 15 min. The colourless supernatant which contained catalase was discarded, and the residue suspended in 100 ml 0.04 *M* phosphate buffer pH 7.0. A solution of trichloracetic acid (10 %, w/v) was added slowly, with stirring, to pH 5.0, and the suspension centrifuged at 20,000 × *g* for 15 min. The deposit contained cytochromes *b* and *c*. The pale yellow supernatant was brought to pH 6.0 with 1 *N* NaOH, and ammonium sulphate (65 g to each 100 ml supernatant) was added with stirring. A copious, white precipitate, which contained cytochromes *a* and *c* and residual catalase, was collected by centrifugation at 20,000 × *g* for 20 min.

The deposit (4.0 g dry wt.) collected after precipitation with trichloracetic acid was suspended in 150 ml 0.04 *M* phosphate, pH 7.0, and again treated with trichloracetic acid as above. The deposit thus obtained (3.2 g dry wt.) was suspended in 100 ml 0.2 *M* acetate buffer, pH 5.0, and 40 mg of Takadiastase was added. The suspension was shaken for 20 h at 35°, and centrifuged at 20,000 × *g* for 15 min. The residue, which contained the cytochrome *b* component and a small quantity of cytochrome *c* was resuspended in 0.2 *M* acetate buffer, pH 5.0, and treated twice more with Takadiastase as above. The final residue (1.5 g dry wt.) contained the cytochrome *b* component and little or no cytochrome *c* (Residue 3, Fig. 1). Absorption maxima at various stages in the procedure are given in Table I.

Attempted solubilization of the cytochrome b *component*

It was found (Fig. 2) that the residues prepared as above dissolved almost completely on treatment with 150 ml 5 % (w/v) "Cetrimide"* (B.P.). Centrifugation

* "Cetrimide" is a mixture of dodecyl, tetradecyl and hexadecyl trimethylammonium bromides.

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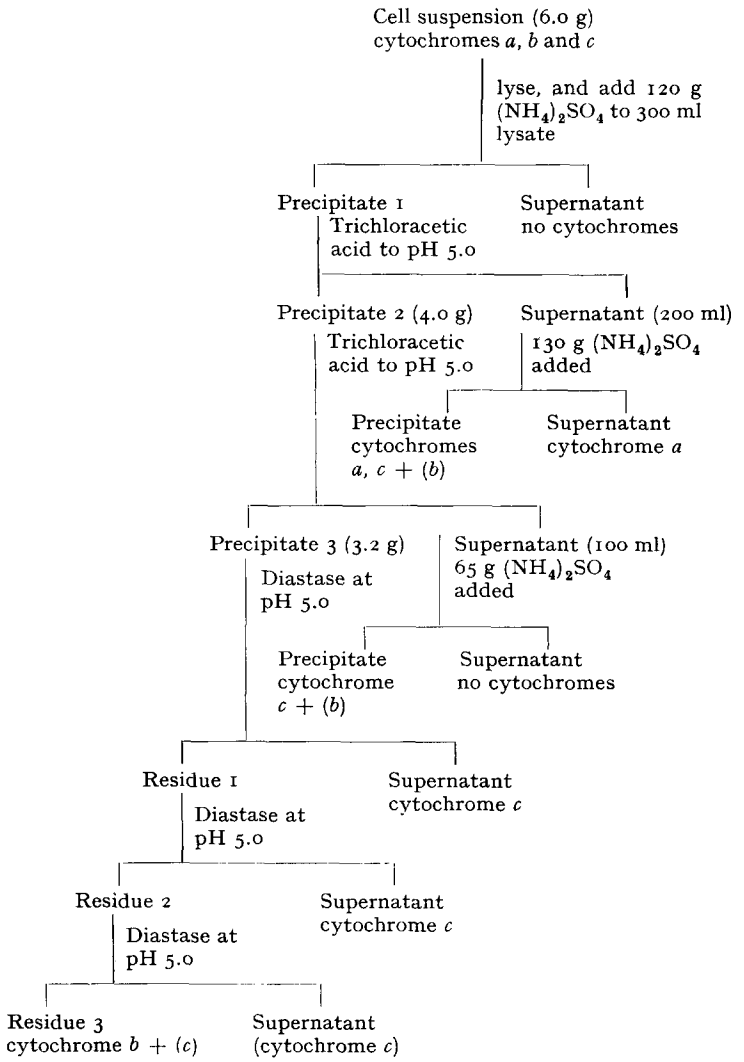

Fig. 1. Separation of the cytochrome *b* component from the other cytochromes. Cytochromes in parentheses were not invariably present in detectable amounts.

at 20,000 × *g* for 15 min separated a small deposit which sometimes contained traces of cytochromes *b* and *c*. The absorption maxima of the reduced component at this stage were still at 560 mμ, 525–530 mμ, and 428–429 mμ (Table I).

Attempts were made to concentrate the cytochrome *b* component by fractional precipitation from the "Cetrimide" solution with ammonium sulphate. To 150 ml of the "Cetrimide" solution were added 30 g ammonium sulphate. At this stage a small amount of yellow precipitate was formed. A further 30 g ammonium sulphate were added and more yellow precipitate formed. This was removed by centrifugation at 20,000 × *g* for 20 min, and spectroscopic examination showed that it contained the small quantity of residual cytochrome *c* together with some cytochrome *b*. To the

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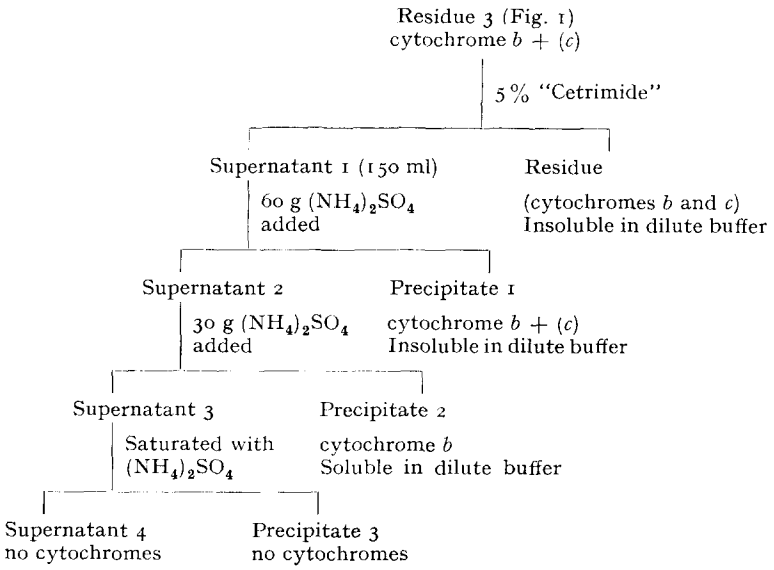

Fig. 2. Stages in the solubilization of the cytochrome *b* component. Cytochromes in parentheses were not invariably present in detectable amounts.

supernatant, a further 30 g ammonium sulphate were added, and a red gelatinous precipitate was obtained (Precipitate 2, Fig. 2) which was collected by centrifugation for 10 min at 20,000 × *g*. This could be dissolved in 0.25 *M* phosphate buffer, pH 7.0, to give a deep amber solution, which became noticeably redder on reduction with dithionite.

Spectroscopic and spectrophotometric examination of this solution showed that the absorption maximum of the *a* band (reduced) had shifted to 558 mμ (Table I).

The yellow pigment of the organism

The concentrated cytochrome *b* preparations derived from the yellow strain of the organism were found to contain considerable quantities of yellow pigment. This pigment was extractable with butanol, but only very small quantities could be extracted with petroleum ether. The absorption maxima in butanol, 429 mμ, 442 mμ and 470 mμ, corresponded to those already reported for a carotenoid pigment isolated from *M. lysodeikticus*[7,8].

The corresponding preparations derived from the "pale" variant of the organism also had associated with them small quantities of yellow pigment. The absorption maxima of this pigment in butanol, 395 mμ, 450 mμ and 470 mμ, differed from those of the pigment from the yellow strain (Fig. 4). Butanol extraction of Residue 3 (Fig. 1) showed that on a weight basis, a "pale" residue contained only about one fifth as much pigment as did a yellow residue.

Stability of cytochrome b *in the preparations*

The cytochrome *b* in Residue 3 (Fig. 1) was stable for at least 14 days after preparation, the material being stored at 4° as the pellet collected by centrifugation.

Solutions of the cytochrome *b*-containing Precipitate 2 (Fig. 2) in 0.04 *M* phos-

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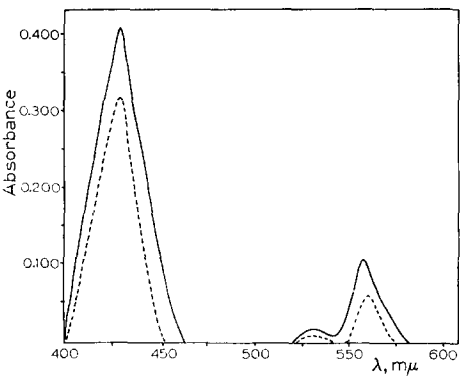

Fig. 3. Absorption spectra of the cytochrome *b* component (reduced preparation read against oxidized preparation). – – – – Residue 3 (Fig. 1) in 50% glycerol – 0.1 *M* phosphate buffer, pH 7.0 — Precipitate 2 (Fig. 2) in 0.1 *M* phosphate buffer, pH 7.0. Readings were taken at 10 mμ intervals, except near peaks, where the intervals were 2 mμ. Negative readings were not plotted.

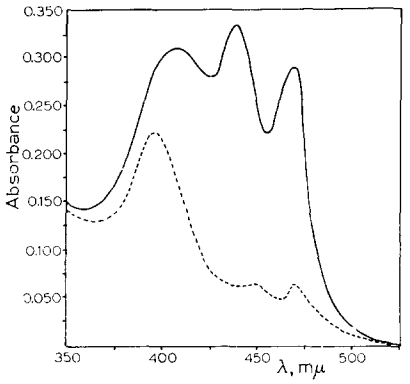

Fig. 4. Absorption spectra of butanol extracts of Residue 3 (Fig. 1). Concentration of extracted material, 350 μg/ml butanol. ——— Extract of Residue 3 derived from yellow strain; – – – – – Extract of Residue 3 derived from "pale" strain. Readings were taken at 10 mμ intervals, except near peaks, where the intervals were 2 mμ.

phate buffer, were dialysed for 24 h against running tap water at room temperature, to remove excess "Cetrimide". During this time precipitation of yellow pigment occurred. The pigment, which was filtered off, was found to contain no detectable cytochromes, but had the absorption spectrum of the carotenoid mentioned earlier. The filtrate was kept at 4° and more yellow pigment separated over a period of 48 h. During the subsequent 72 h, however, no further precipitation occurred.

If the remaining solution was evaporated to dryness over P_2O_5, a waxy orange-red solid was obtained, which could be re-dissolved almost completely in 0.1 *M* phosphate buffer pH 7.0. This contained the 558 mμ-absorbing material, which was stable for at least 6 months in the dry form. On the other hand, in solutions which had been kept at 4° for 2 weeks, a new absorption band was seen at 570 mμ after reduction, and within 1 month, the 558 mμ band was no longer visible, but another new band had appeared at 552–554 mμ. This change in absorption characteristics was presumably due to formation of degradation products of cytochrome *b*, perhaps analogous to those reported[9,10] in preparations of mammalian cytochrome *b*.

The cytochrome *b* component obtained from the "pale" strain of the organism became degraded similarly under these conditions, although there was no precipitation of yellow pigment at any stage.

Removal of most of the yellow pigment by repeated butanol extractions of the insoluble preparations or solubilized materials seemed to have a deleterious effect on the cytochrome *b*, as the α band of the cytochrome was fainter and less well defined than before this treatment.

Several extractions of Residue 3 (Fig. 1) with 1% (w/v) sodium deoxycholate, or a single extraction with a 2% (w/v) solution, solubilized considerable quantities of the yellow pigment, without apparently damaging the cytochrome *b*, most of which remained associated with the residue. This residue dissolved readily in (5% w/v) "Cetrimide", and on reduction the solution showed an α band with a maximum

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at 560 mμ. Further experiments showed that "Tween 80" (2 % v/v) could be used instead of deoxycholate, with similar results.

The best preparations of cytochrome *b* were obtained from organisms or lysates which had not been stored (4°) for more than 7 days after harvesting. If organisms or lysates which had been kept for longer than this were used, the final preparations were found to have an additional absorption band at about 570 mμ.

Oxidation and reduction of the cytochrome b

The cytochrome *b* in Residues 1, 2 and 3 (Fig. 1) was in the oxidized state; after reduction with minimal quantities of dithionite, it could be re-oxidized by shaking with air. Re-oxidation of the cytochrome *b* in Residue 3 (0.1 *M* phosphate, pH 7.0), was not prevented by the addition of cyanide to final concentration $5.0 \cdot 10^{-2}$ *M*, or by 2-heptyl-4-hydroxyquinoline-N-oxide ($1.5 \cdot 10^{-4}$ *M*).

Samples of Residue 3 (Fig. 1) in 0.1 *M* phosphate buffer pH 7.0, were placed in Thunberg tubes, and after evacuation and flushing four times with oxygen-free nitrogen, succinate was added from the stopper to concentration 0.036 *M*. No reduction of the cytochrome *b* was observed, even after 32 h at 30°.

Under similar anaerobic conditions, the cytochrome *b* of Residue 3 (Fig. 1), suspended in 0.1 *M* phosphate buffer, pH 7.0, was not reduced by potassium ferrocyanide, final concentration 0.01 *M*. At the same concentration and pH, sodium ascorbate produced no immediate reduction, but after 5 min a faint band of reduced cytochrome *b* was visible, and this did not increase much in density in the course of 2 h at room temperature. In similar experiments, each of these reagents was found to reduce mammalian cytochrome *c* within a few seconds of mixing.

TABLE I

ABSORPTION MAXIMA OF REDUCED CYTOCHROMES AT VARIOUS STAGES OF THE EXTRACTION PROCESS

Observation on whole cell suspensions were made by visual spectroscopy. The other materials were examined both spectrophotometrically and visually. Cytochromes in parentheses were not invariably present in detectable amounts.

Material	*Absorption maxima (mμ)*				
	α		*β*		*γ*
	Room temperature	*Liquid oxygen*	*Room temperature*	*Liquid oxygen*	*Room temperature*
Cell suspension	595, 561, 550	595, 560, 550	525–530	530	
Lysate	595, 561, 550	595, 560, 550	525–530	525–530	429
Precipitate 1 (Fig. 1)	595, 560–563, 550		525–530		
Precipitate 2 (Fig. 1)	595, 560–563, 550		525–530		
Residue 1 (Fig. 1)	560–563, 550		525–530		
Residue 3 (Fig. 1)	560, (550)	560, (550)	525–530	525–530	429–430
Supernatant 1 (Fig. 2)	560	560	525–530	525–530	428–429
Precipitate 1 (Fig. 2)	(560), 550	(560), 550	525–530	525–530	
Precipitate 2 (Fig. 2)	558	558	530	530	430

A sample of Precipitate 2 (Fig. 1) was washed with distilled water and resuspended in 0.1 *M* phosphate buffer, pH 7.0. On addition of sodium ascorbate to concentration 0.015 *M*, under anaerobic conditions, marked reduction of cytochrome *c* in the preparation occurred within a few seconds, but although the band of reduced cytochrome *b* could be detected after about 5 min, it remained very faint. When potassium

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ferrocyanide (final concn. 0.015 *M*) was added, instead of ascorbate, to a similar preparation, there was no immediate reduction of cytochromes *b* or *c*. After 30 min at room temperature, the band of cytochrome *c* was seen, but the cytochrome *b* was not reduced.

DISCUSSION

The solubilization of two bacterial *b*-type cytochromes was reported by VERNON[11], who found that these components of *Micrococcus denitrificans* and *Pseudomonas denitrificans* could fairly easily be obtained in solution. By the application of similar methods to *M. lysodeikticus*, we failed to solubilize the cytochrome *b* component, which remained attached to particles. The separation of heart-muscle cytochrome *b* from particulate material by means of sodium deoxycholate has been achieved by BERNSTEIN AND WAINIO[10]. We have found that treatment with sodium deoxycholate alone or Tween 80, while useful for removal of excess carotenoid pigment, did not lead to adequate solubilization of the cytochrome *b* component of *M. lysodeikticus*.

In the intact *M. lysodeikticus* and lysates, the α-absorption band of the reduced cytochrome *b* was broad, and appeared on visual spectroscopic examination to extend from about 555 mμ to 565 mμ. The maximum was difficult to determine visually, but in liquid oxygen-cooled preparations appeared to be at 560 mμ. Table I gives the absorption maxima of the bands determined at different stages of the extraction process, at room temperature and after cooling in liquid oxygen. The absorption maximum of the α-band of the reduced cytochrome *b* was at about 560 mμ, except in Precipitate 2 (Fig. 2). The precipitation of this material from the "Cetrimide" solution with ammonium sulphate produced a shift in the absorption maximum from 560 mμ to 558 mμ; this is shown too in Fig. 3. It is possible that very slight shifts occurred in the maxima of the β- and γ-bands.

In an attempt to ascertain the cause of the shift, it was found that treatment with 5 % "Cetrimide" alone, even on standing for 20 days at 4°, did not affect the absorption maximum of mammalian cytochrome *c*, nor the maxima of cytochromes *b* and *c* in the crude *Micrococcus* lysates and cytochrome *b*-containing residues. Stabilization of pH at 7.0 during the precipitation did not prevent the change. The possibility that the broad cytochrome *b* band might be due to the fused bands of 2 components, and that in the fractionation of the solubilized material one only of these was present in Precipitate 2 (Fig. 2), was not supported by further observations. The cytochrome *b* band in reduced whole-cell suspension, lysates and Residue 3 (Fig. 1) was not split by cooling in liquid oxygen. Furthermore, no cytochromes were detected in precipitates obtained by full ammonium sulphate saturation of the "Cetrimide" solution after removal of Precipitate 2 (Fig. 2).

There is a further possibility that, at the stage of precipitation from the "Cetrimide" solution a haemochromogen might have been formed as a result of reaction of the cytochromes with an impurity in the preparation.

In the fractionation procedures, some carotenoid pigment was found to accompany the cytochrome *b*, and separation of the two without degradation of the cytochrome was not achieved. The difficulty of extracting the carotenoid with petroleum ether suggests that some of it might be protein-bound, and it is possible that it

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is bound to the protein moiety of cytochrome *b*. Attempts at separation on ion-exchange materials and by differential absorption on kaolin have not so far been successful. It is interesting that cytochrome *b* preparations obtained from the "pale" variant also contained small amounts of a yellow pigment, although the absorption spectrum of this differed from that of the carotenoid from the parent N.C.T.C. 2665 strain.

The observations on reduction and oxidation of the *b* and *c* cytochromes of *M. lysodeikticus* suggest that the potential of the *b* component is lower than that of the bacterial and mammalian cytochrome *c*, and are consistent with a cytochrome sequence in which the *b* component precedes *c* as in the mammalian electron-transport system. Like VERNON's solubilized cytochromes, the cytochrome *b* component of our preparations was "auto-oxidizable" in the presence of cyanide, but without further purification it is not possible to say that no electron acceptor intervenes between the cytochrome *b* and oxygen.

The absorption characteristics of the cytochrome *b* component of *M. lysodeikticus* are in many respects similar to those of the so-called "cytochrome b_1" of other organisms. The nomenclature (*b*, b_1), is based on small spectroscopic differences, the exact significance of which is uncertain[12], and for the present we have not assigned the *M. lysodeikticus* *b*-type cytochrome to a definite subgroup.

ACKNOWLEDGEMENT

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