muscle extract used as the starting material. On spectrophotometric examination the solution may show a slight absorption peak at 553 mμ which is absent if the solution is treated with ferricyanide. Addition of dithionite produces a strong absorption band at 562 mμ and a shoulder centered at 553 mμ. Succinate causes only a partial reduction of the material absorbing at 562 mμ and complete reduction of the material absorbing at 553 mμ. Addition of ascorbic acid selectively produces the 553-mμ band with little change at 562 mμ. The preparation is rich in succinic dehydrogenase but apparently devoid of cytochrome c. Addition of succinate and cytochrome c to the preparation causes immediate reduction of the cytochrome c.

It is concluded that cytochrome b is the predominant hemoprotein present in the preparation, but that at least one other hemoprotein, tentatively designated cytochrome 553, is also present. The role of the latter component in the linking of succinic dehydrogenase with cytochrome c is not yet clear. The positions of the absorption bands of the reduced components are as follows:

	α	β	γ
Cytochrome b	562 mμ	530 mμ	430 mμ
Cytochrome 553	553 mμ	522 mμ	417 mμ

[132] Cytochrome b Group (Bacteria)

By A. M. Pappenheimer, Jr.

The heme-containing pigments which have been called cytochrome b represent a group of proteins which not only differ from one another in physicochemical properties but probably differ as well in their catalytic function in the chain of respiratory enzymes. Thus, although all members of the b group show α and β absorption bands in the visible spectrum and a strong γ or Soret band in the violet region, the exact position of these bands varies from one bacterial species or strain to another. In the table, the positions of the α, β, and γ bands for the cytochrome b components of certain representative bacterial species and for yeast are given. It can be seen that they may be classified into groups according to the position of their α bands.

1. Cytochrome b with absorption maximum 562 to 565 mμ.
2. Cytochrome b_1 at 560 mμ.
3. Cytochrome b_2 of yeast at 556 mμ.
4. A component absorbing at 554 mμ which may or may not belong to this group and which is characteristic of *Acetobacter*.[1]

[1] L. Smith, *Bacteriol Revs.* **18**, 106 (1954).

Finally, the recently discovered cytochrome e[2] with an α band at 552 to 553 mμ at liquid air temperature is present in *Bacillus lichenformis* as well as in yeast and various animal and insect tissues. Recent studies suggest that cytochrome e should be considered with the b group.[3]

DIFFERENCE SPECTRA OF CYTOCHROME b IN CERTAIN BACTERIA[a]

Strain	Position of maxima for reduced pigment, mμ			Type
	α	β	γ	
Baker's yeast	564	525	430	b
Bacillus subtilis	564	523	422	
Staphylococcus albus	565	523	427	
Sarcina lutea	562	523	430	
Aerobacter aerogenes	560	530	430	b_1
Escherichia coli	560	533	432	
Azotobacter chroococcum	560	530	428	
Corynebacterium diphtheriae[c]	560	524	429	
Acetobacter pasteurianum	554	523	428	?
Acetobacter suboxydans	554	525	422	
Delft yeast[d]	557	528	424	b_2

[a] This table has been constructed from the data of Smith.[b] Except for the bands of *C. diphtheriae* and of yeast, all values are for difference spectra, i.e., difference in extinction between oxidized and reduced pigments.

[b] L. Smith, *Bacteriol. Revs.* **18,** 106 (1954).

[c] A. M. Pappenheimer, Jr., and E. D. Hendee, *J. Biol. Chem.* **171,** 701 (1947).

[d] C. A. Appleby and R. K. Morton, *Nature* **173,** 749 (1954).

In addition to the similarity in their absorption spectrum, the various members of the b group have certain other characteristics in common. Most of the cytochrome b's appear to be autoxidizable at an appreciable rate, although far more slowly than is cytochrome oxidase. They do not combine with CO, HCN, or HN_3, although all the group probably contain protoheme as their prosthetic moiety. Their oxidation potential lies below that of cytochrome c, and in the few cases studied their position in the respiratory chain lies below that of cytochrome c.

Little is known of the function of the bacterial cytochrome b group. Cytochromes b and b_1 are both concerned in the oxidation of succinate.[4,5]

[2] D. Keilin and E. F. Hartree, *Nature* **164,** 254 (1949).

[3] A. M. Pappenheimer, Jr., and C. M. Williams, *J. Biol. Chem.* **209,** 915 (1954).

[4] D. Keilin and C. H. Harpley, *Biochem J.* **35,** 688 (1941).

[5] A. M. Pappenheimer, Jr., and E. D. Hendee, *J. Biol. Chem.* **180,** 597 (1949).

Cytochrome b_2 of yeast appears to be closely associated with lactic dehydrogenase activity (see Vol. I [68] for preparation and properties of yeast lactic dehydrogenase). The cytochrome b_1 of *E. coli* is not only involved in succinate oxidation[4] but may also be an integral part of the nitrate reductase system.[6]

None of the cytochrome b group has been isolated in pure form as a soluble pigment with the possible exception of cytochrome b_2 of yeast.[7,7a] All other members appear to be associated with large mitrochondrial and microsomal fragments.

Preparation and Partial Purification of Cytochrome b_1 from *Corynebacterium diphtheriae*[5]

Bacterial Cultures. The Toronto strain of *Corynebacterium diphtheriae* is grown in 5- to 10-l. lots in Povitsky bottles as a pellicle on the surface of Mueller and Miller's casein hydrolyzate medium[8] containing 1.5% maltose. Just before inoculation 25 mg. of $FeSO_4 \cdot 7H_2O$ per liter is added as a sterile solution. The pH of the medium should be below 7.5 at the time of harvesting. After 6 to 7 days' growth at 34°, the bacteria are collected by centrifugation and are washed three times with saline in the centrifuge.

Extraction of Cytochrome b_1 from the Bacterial Cells. Washed cells from 5 l. of culture are suspended in 150 to 200 ml. of saline, and the thick suspension (6 to 8 mg. bacterial N per milliliter) is disrupted for 30 minutes in a 9000-cps sonic oscillator (Raytheon Corp., Waltham, Massachusetts) in 25-ml. charges.

If a sonic oscillator is not available, the cells may be broken up by grinding.[9]

Partial Purification of Cytochrome b_1. The purification procedure by differential centrifugation is entirely empirical, and no rigid procedure can be outlined.

The Q_{O_2} (succinate, KCN) and Q_{O_2} (succinate, MB, KCN) were 146 and 4070 μl./mg. N/hr., respectively, for a crude diphtherial extract containing 7.6 mg. N/ml. The purification of this particular lot was accomplished as follows: 180 ml. of extract was centrifuged for 30 minutes at 10,000 r.p.m. in a refrigerated International centrifuge to remove bacterial debris. The turbid supernatant was then centrifuged at 12,000 r.p.m. (ca. 18,000 $\times$ g) in a Servall centrifuge, Model SS-2, for 30 minutes. Then

[6] R. Sato and F. Egami, *Bull. Chem. Soc. Japan* **22,** 137 (1949).
[7] S. J. Bach, M. Dixon, and L. C. Zerfas, *Biochem. J.* **40,** 229 (1946).
[7a] C. A. Appleby and R. K. Morton [*Nature* **173,** 749 (1954)] have isolated the lactic dehydrogenase of yeast as a crystalline hemoprotein containing flavin.
[8] J. H. Mueller and P. Miller, *J. Immunol.* **40,** 21 (1941).
[9] G. Kalnitsky, M. F. Utter, and C. H. Werkman, *J. Bacteriol.* **49,** 595 (1945).

25 ml. of fatty upper layer of low activity was removed and discarded. Next 130 ml. of a clear red layer containing more than 60% of the total succinic oxidase activity was removed from a small amount of sediment and centrifuged in the Servall at 16,000 r.p.m. (ca. 30,000 × *g*) for 3 hours. Once again three layers were obtained: an inactive yellow upper layer, a clear reddish-brown layer with considerable activity, and a highly active dark wine-red transparent sediment at the bottom of the tube. The sediment was suspended in 0.02 *M* phosphate buffer, pH 7, and homogenized for 20 minutes in the sonic oscillator. Thirty-five milliliters of dark-red solution was obtained which was clarified by centrifugation in the Servall for 30 minutes at 12,000 r.p.m. This solution contained about 30% of the total original succinic oxidase activity. Its Q_{O_2} (succinate, KCN) was 440 and Q_{O_2} (succinate, MB, KCN) was 14,000 μl. O_2/mg. N/hr. This represents a purification of 3- to 3.5-fold, on the basis of both hemin content and specific activity.

Properties

The above preparation contained 3.9 mg. N/ml. and 1.09 γ hemin per milligram of nitrogen. Solutions of the purified pigment are dark red in color, clear by transmitted light, but show a pronounced Tyndall effect. After reduction with dithionite or with succinate, the color changes to a more pinkish hue and intense absorption bands centered at 560 and 529 mμ become readily visible with a hand spectroscope. The Soret band of the oxidized pigment shows a maximum absorption at 415 mμ which shifts to 429 mμ on reduction.

When examined in the ultracentrifuge, cytochrome b_1 is polydisperse, and no distinct sedimenting components can be identified. On standing, even in the cold, aggregation occurs, and within 1 or 2 days solutions become turbid without, however, appreciable loss in enzymatic activity. Thus, although cytochrome b_1 cannot be regarded as a truly soluble protein such as cytochrome c, freshly purified preparations, in contrast to similarly prepared material from mammalian tissues, yield clear solutions which can readily be studied in an ordinary Beckman spectrophotometer.

The preparations can be dried from the frozen state or can be precipitated at pH 4.8 in the cold without loss of activity. However, reconstitution of dried or precipitated b_1 in buffer always yields turbid solutions.

When purified preparations are heated to 60° at pH 7.6, aggregation occurs and the solution rapidly becomes turbid. Actual coagulation does not occur until 75°, at which temperature 75% of the succinic oxidase activity is lost in 30 minutes.

Assay of Enzymatic Activity. Diphtherial cytochrome b_1 preparations

catalyze the oxidation of succinate to fumarate. Unlike the b_1 of *E. coli*,[4] the reaction is not affected by $M/400$ KCN. Since available evidence suggests that for the diphtherial system under these conditions autoxidation of b_1 is the rate-controlling step, manometric determination of succinic oxidase activity in the presence of cyanide can be used as a method of assay.

The total volume in each Warburg vessel is 2.2 ml., including 0.2 ml. of 20% NaOH in the center well and 0.2 ml. of $M/5$ sodium succinate in the side arm. Phosphate buffer, pH 7.8, to give a final concentration of $M/10$, 0.1 ml. of $M/20$ KCN, and a suitable quantity of enzyme are placed in the vessel itself. Control vessels without substrate should always be included but usually show no oxygen uptake. Q_{O_2} values may be calculated from the oxygen consumed during the first 30 minutes and expressed as microliters per milligram of nitrogen per hour. The enzyme shows a sharp pH optimum at 7.8.

Other Methods of Assay. Since succinic oxidase activity as measured above has always proved proportional to succinic dehydrogenase activity, any method for measuring the latter (see Vol. I [121]) would appear suitable for assay of diphtherial cytochrome b_1. Succinic dehydrogenase may be determined manometrically in the presence of methylene blue and cyanide or by the anaerobic ferricyanide method of Quastel and Wheatly.[10] Both methods are more sensitive than the one described above, because the autoxidation of methylene blue is more rapid than that of cytochrome b_1 and because oxidation of reduced b_1 by either methylene blue or ferricyanide is more rapid than its autoxidation by molecular oxygen.

$$Q_{CO_2}^{N_2}(\text{succ., ferricyanide}) = 4Q_{O_2}(\text{succ., MB, KCN}) = 120Q_{O_2}(\text{succ., KCN})$$

Succinate oxidation may also be determined spectrophotometrically by following the rate of reduction of the nonautoxidizable dye 2,6-dichlorophenolindophenol (Price and Thimann).[11]

[10] J. H. Quastel and A. H. M. Wheatley, *Biochem. J.* **32,** 936 (1938).
[11] C. A. Price and K. V. Thimann, *Arch. Biochem. and Biophys.* **33,** 170 (1951).