SUCCINOXIDASE SYSTEM OF *PASTEURELLA TULARENSIS*

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The succinoxidase system of *Pasteurella tularensis* has been studied as part of a general investigation on the metabolism of this organism. This paper describes some of the properties of the intact system; data on the components of the succinic dehydrogenase system are presented elsewhere (Wadkins and Mills, *unpublished data*).

EXPERIMENTAL METHODS

Preparation of washed particles. Cell-free extracts of highly virulent *P. tularensis* strain Sm were prepared by sonic disintegration of cells as described by Rendina and Mills (1957). Twenty ml of undialyzed centrifuged (3000 × G for 30 min) sonic extract, containing 3.8 to 4.2 mg N per ml, were centrifuged for 3 hr at 105,000 × G at 5 C. The supernatant was decanted and the reddish precipitate was suspended in 20 ml 0.1 M potassium phosphate buffer, pH 7.5, and recentrifuged 1 hr at 2000 × G. The precipitate, after an additional washing with phosphate buffer, was called "washed particles."

Alcohol fractionation of sonic extract. Fifty ml of sonic extract, after dialysis overnight against 1 L distilled water at 4 C, were adjusted to pH 5.4 with acetate buffer. The precipitate was separated by centrifuging at 3000 × G, resuspended in distilled water, and the pH adjusted to 6.5 with bicarbonate buffer. Ethanol, 95 per cent, was added slowly to a final concentration of 20 per cent, while maintaining the temperature at or below −2 C. The precipitate was centrifuged off at 3000 × G at −5 C, taken up in water, and lyophilized. This material contained all of the cytochrome *b* as well as the succinoxidase activity of the sonic extract.

Oxygen consumption was measured by conventional techniques in the Warburg respirometer at 37 C, with air as gas phase. Final reaction volume was 3.0 ml, at pH 7.4. Succinoxidase activity was also determined by measuring accumulation of fumarate as indicated by increase in optical density at 240 mμ (Racker, 1950).

Ethyl hydrogen peroxide was prepared by the procedure detailed by Stern (1936).

RESULTS

Whole cells and sonic extracts of *P. tularensis* possess an active succinoxidase system. After 20 ml of sonic extract were centrifuged 4 hr at 25,000 × G at 5 C, the activity was found almost entirely in particulate fractions. Heavy particles (a pink precipitate on the bottom of the tube) had a Q_{O_2} somewhat higher than the original sonic extract (table 1). Just above the precipitate was a heavy cherry-red solution which was overlaid by clear yellowish supernatant. These solutions were decanted together and centrifuged for 7 hr as before. The red solution, apparently containing small particles, was separated from the yellow supernatant, and was found to have a high succinoxidase activity. The yellow supernatant was only slightly active. When sonic extract was centrifuged at 144,000 × G, similar to the preparation of "washed particles" under Experimental Methods, most of the succinoxidase activity was in the sediment.

The difference spectrum of a sonic extract of *P. tularensis* was determined in the spectrophotometer, using a few crystals of sodium hydrosulfite to reduce the cytochromes. The peaks indicated the presence of a *b*-type cytochrome (429, 530, 559 mμ), cytochrome a_2 (628 mμ), and a suggestion of cytochrome a_1 (a shoulder at 590 mμ). There was no evidence for the presence of a *c*-type cytochrome, or of a_3. Similar but more sharply defined spectra were obtained with deoxycholate-treated (2 per cent) sonic extract, and with a small particle preparation (Wadkins and Mills, *unpublished data*) (figure 1). Examination of sonic extracts at the temperature of liquid air with a hand spectroscope did not reveal any cytochrome *c* bands.

Using the method of Keilin (1926), no cytochrome *c* was obtained by extraction of 10 g wet weight of *P. tularensis*. A parallel experiment

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with baker's yeast gave large amounts of cytochrome *c*.

The addition of horse heart cytochrome *c* (1 × 10^{-4} M final concentration) had no effect on the succinoxidase activity of a sonic extract. However, added cytochrome *c* was slowly reduced (measured at 550 mμ) by sonic extract in the presence of succinate; 10^{-3} M cyanide inhibited this reduction after 5 min contact with the enzyme.

Both whole cells and sonic extracts were devoid of cytochrome oxidase activity, as determined by reoxidation of reduced cytochrome *c* (Cooperstein and Lazarow, 1951), and by oxidation of *p*-phenylenediamine or Nadi reagent. In control experiments dilute rat liver homogenate was very active in both assays.

TABLE 1

Succinoxidase activity and cytochrome b content of centrifugal fractions

Fraction	Cytochrome *b*	Q_{O_2}
Whole sonic extract	+	139
Yellow supernatant	−	24
Red supernatant	++++	544
Precipitate	++	238

Cytochrome *b* estimated with hand spectroscope. Q_{O_2} is μLO_2/mg N/hr. Each vessel contained 100 μmoles succinate, 150 μmoles potassium phosphate buffer, and the material to be assayed.

The relative concentrations of cytochrome *b* in the centrifugal fractions of the sonic extract, as estimated with a hand spectroscope after reduction with hydrosulfite, correlated well with the succinoxidase activity (table 1).

The addition of succinate (0.05 M final concentration) to sonic extract in an evacuated Thunberg tube caused the immediate appearance of the 560 mμ band of reduced cytochrome *b* as seen with the hand spectroscope. Upon admission of air to the tube, the band rapidly faded, but did not disappear, indicating partial reoxidation of the cytochrome *b*.

The concentration of the cytochrome *b* in the sonic extract as determined by optical density change at 560 mμ upon reduction with hydrosulfite, using the value 22 $cm^{-1}mM^{-1}$ for the change of molecular extinction coefficient (Chance, 1957), was about 4 × 10^{-6} M, or 1 × 10^{-3} μmoles per mg N.

The cytochrome *b* was thermolabile, as indicated by nonreducibility with succinate or hydrosulfite after incubation for 20 min at 80 C. Heating a tube containing enzymatically reduced cytochromes *b* and *c* for 40 min at 75 C resulted in disappearance of the reduced cytochrome *b*, whereas the reduced cytochrome *c* was unchanged.

Sonic extracts reduced methylene blue in an evacuated Thunberg tube in the presence of succinate; upon tipping in fumarate the methylene

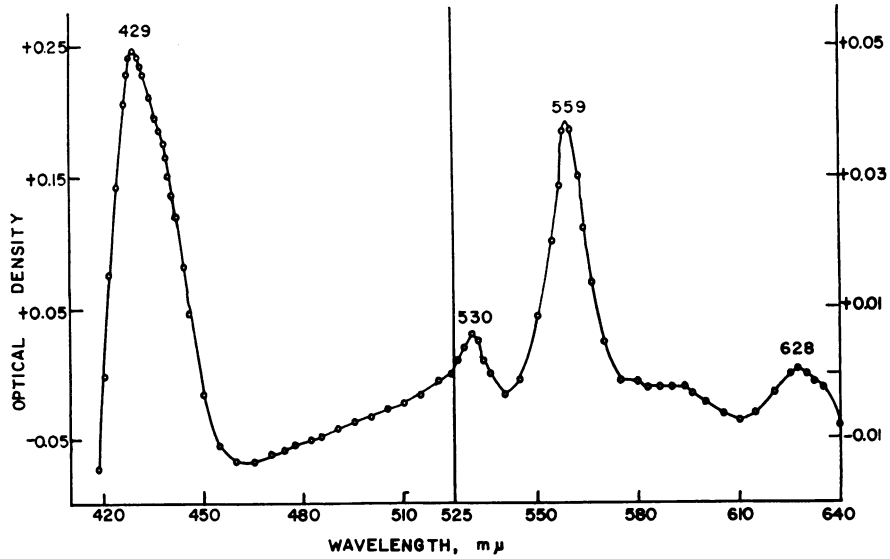

Figure 1. Difference spectrum of small particle preparation of *Pasteurella tularensis*

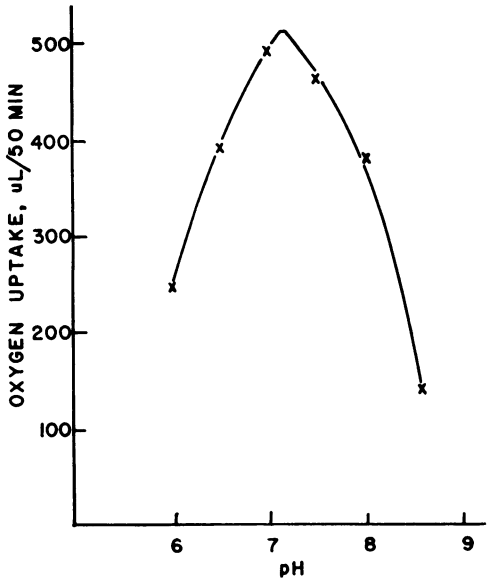

Figure 2. Effect of pH on succinoxidase activity of sonic extract. Each flask contained succinate, 100 μmoles; 0.1 M phosphate buffer, 1 ml; sonic extract, 1 ml.

blue was reoxidized, as would be expected. The reduced cytochrome *b* band which had been produced in sonic extract by the addition of succinate disappeared completely and rapidly after addition of methylene blue. Methylene blue, 1.8×10^{-4} M, had little effect on oxygen uptake by sonic extract with succinate, and did not reverse the inhibition of oxygen uptake produced by cyanide (1×10^{-3} M). Since the methylene blue can reoxidize the cytochrome *b*, it appears that the rate of reoxidation of cytochrome *b* is not rate limiting in the succinoxidase system.

Properties of the succinoxidase. The effect of pH on the succinoxidase activity is shown in figure 2. At pH 7.3, the maximal rate of oxygen uptake occurred.

When the partially purified succinoxidase preparation obtained by alcohol fractionation was dialyzed 24 hr against distilled water, there was complete loss of succinic dehydrogenase and succinoxidase activity. Known coenzymes such as di- and triphosphopyridine nucleotide, riboflavin, riboflavin phosphate, and flavin adenine dinucleotide did not restore activity, but in the presence of 3.3×10^{-3} M cysteine the activity was 85 per cent that of the undialyzed preparation. Dialysis had no effect on the activity of the original sonic extract.

TABLE 2

Effects of cysteine and ascorbic acid on succinoxidase

Additions	Oxygen Uptake
	μL/30 min
None	6
Succinate	187
Succinate + cysteine	186
Succinate + ascorbate	188
Cysteine	19
Ascorbate	16

Each vessel contained 0.5 ml sonic extract and 150 μmoles potassium phosphate buffer. Final concentrations of additions: succinate, 1×10^{-2} M; cysteine, 3.3×10^{-3} M; ascorbate, 3.3×10^{-3} M.

A component of mammalian succinoxidase system is inhibited by reducing agents such as cysteine, ascorbic acid, glutathione, and British Anti-Lewisite (Slater, 1949), and by antimycin A (Potter and Reif, 1952). Cysteine and ascorbic acid (table 2) and antimycin A (5 μg per ml) all had no effect on the succinoxidase activity of sonic extracts of *P. tularensis*.

Attempts to demonstrate hydrogen peroxide production during the oxidation of succinic acid by sonic extracts or by washed particles were unsuccessful. Since the organism and sonic extracts contain high catalase activity, the peroxidative oxidation of ethyl alcohol (Keilin and Hartree, 1945) was attempted. The presence of 3.3×10^{-2} M ethyl alcohol had no effect on oxygen uptake, and no acetaldehyde could be detected in the medium.

A specific catalase inhibitor, ethyl hydrogen peroxide, caused marked inhibition of oxygen uptake (95 per cent at 7×10^{-2} M, 30 per cent at 7×10^{-3} M). However, the ethyl hydrogen peroxide was apparently acting as a direct oxidant of the system. When added to a succinate-reduced system in an evacuated Thunberg tube, it caused complete disappearance of the reduced cytochrome *b* band. That peroxide could reoxidize the oxidase system enzymatically was indicated by the effect of 1×10^{-3} M hydrogen peroxide on the reduction of 2,6-dichlorophenolindophenol by washed particles (figure 3). The marked inhibition of dye reduction by the peroxide was prevented by the presence of 3.3×10^{-4} M or 3.3×10^{-5} M cyanide, and partially reversed by the addition of cyanide after reduction of the

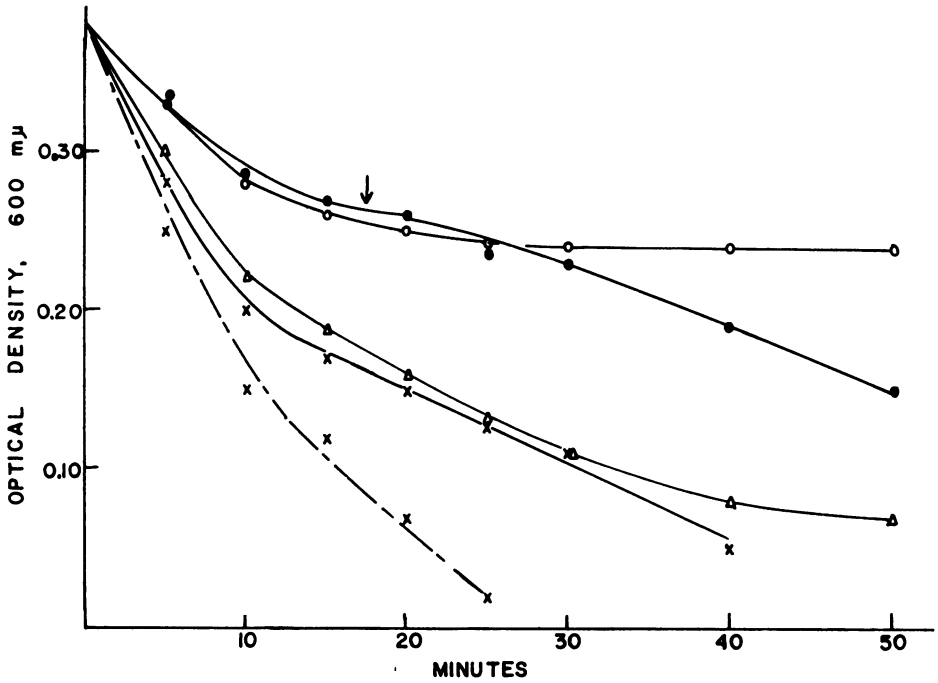

Figure 3. Effect of H_2O_2 and NaCN on reduction of 2,6-dichlorophenolindophenol. Each tube contained 0.1 ml sonic extract (0.4 mg N), 2.7×10^{-5} M 2,6-dichlorophenolindophenol, 0.01 M sodium succinate (except for endogenous tube), and 0.08 M potassium phosphate, pH 7.4. Additions: ×——× none; ×---×, 5×10^{-4} M NaCN; ○——○ 1×10^{-3} M H_2O_2; △——△ H_2O_2 + NaCN; ●——● H_2O_2, with NaCN added at arrow; 26 C; enzyme added at zero time.

dye had stopped completely in the presence of the peroxide.

Cyanide (1×10^{-3} M) and sulfide (2×10^{-3} M) inhibited markedly the succinoxidase activity as followed by oxygen uptake (table 3). Most of the oxygen consumption in the presence of cyanide was the result of a delay of onset of inhibition after mixing the cyanide with the washed particles. This lag is illustrated in figure 4, where fumarate production was measured in the presence of a very small amount of sonic extract. Added cysteine had little effect on the inhibition by cyanide, but completely reversed the inhibition caused by sulfide (table 3).

These effects were observed also in the growth of the organisms in the casein hydrolyzate-decamin broth, which contained high levels of cysteine (1.3×10^{-2} M). During growth large amounts of hydrogen sulfide are produced from the cysteine, with no apparent effect on the growth. Added sodium sulfide (2×10^{-2} M) had no effect on the rate of growth, but 2×10^{-4} M cyanide completely inhibited growth. Sodium azide at 1.5×10^{-4} M caused 20 per cent inhibition of growth, and complete inhibition at 7.5×10^{-4} M.

TABLE 3

Effects of sulfide and cyanide on succinoxidase

Additions to Flasks	Oxygen Uptake
	μL/30 min
None	0
Succinate	201
Succinate + sulfide	78
Succinate + sulfide + cysteine	212
Cysteine	0
Cysteine + sulfide	22
Succinate + cyanide	104
Succinate + cyanide + cysteine	129
Succinate + cysteine	212

Each flask contained 0.5 ml washed particle suspension plus 150 μmoles potassium phosphate buffer. Final concentrations of additions were: succinate, 7×10^{-3} M; cysteine, 3.3×10^{-3} M; sodium sulfide, 2×10^{-3} M; sodium cyanide 1×10^{-3} M.

It was of some interest to determine whether the presence of sulfide in the growth medium

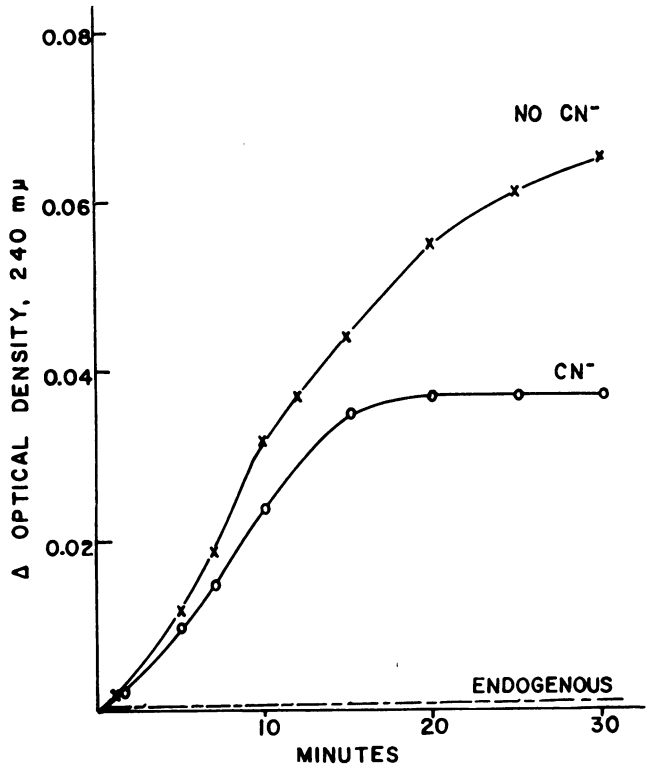

Figure 4. Effect of cyanide on rate of fumarate production by sonic extract. Final concentrations in cuvettes: succinate, 3.3×10^{-2} M; cyanide, 5×10^{-3} M, potassium phosphate, 6.7×10^{-2} M; 0.001 ml sonic extract. Total volume 3.0 ml. Endogenous had no succinate or cyanide. Enzyme added at zero time.

would have any effect on the cytochrome composition of a microorganism. *P. tularensis* is difficult to grow in the absence of added cysteine and therefore in the absence of sulfide, but *Bacillus subtilis* grows well on nutrient agar. After 24 hr growth of *B. subtilis* on nutrient agar with and without 0.1 per cent added sodium sulfide, the cells were washed once, and resuspended in saline. After addition of hydrosulfite the reduced spectra were examined with a hand sepectroscope. The cells grown without added sulfide showed the typical 3 bands at 530, 550–562, and 600–604 mμ. The organisms grown in the presence of sulfide showed only a much fainter single broad band at 551–559 mμ, with no visible band at 595–604 mμ. While the organisms grown in the absence of sulfide contained cytochrome oxidase activity (Nadi reagent), those grown with added sulfide had none.

DISCUSSION

Succinoxidase systems in a number of microorganisms have been studied with a varying magnitude of enterprise; wherever information on intracellular distribution is available the activity has been shown to be contained in the particulate fractions. *Mycobacterium phlei* (Brodie and Gray, 1956) and *Mycobacterium avium* (Kusunose *et al.*, 1956) may appear to be exceptions, since the "soluble" fraction is also required for succinoxidase activity. However, the "soluble" fractions may simply remove inhibitory products as suggested by Kusunose, or may contain products of partial fragmentation of the succinoxidase complex resulting from prolonged sonic treatment.

It is interesting that in every microorganism with succinoxidase activity where there is information on the cytochrome composition of the organism, a *b*-type cytochrome (*b*, 564 mμ, or

b_1, 560 mμ) is present. Micoorganisms in which this is true include, in addition to *P. tularensis*, the following: *Aerobacter aerogenes* (Tissieres, 1952), *Azotobacter agilis* (*A. vinelandii*) (Bruemmer *et al.*, 1957; Tissieres, 1956), *Bacillus subtilis* (Smith, 1954*a*), *Corynebacterium diphtheriae* (Pappenheimer and Hendee, 1947), *Escherichia coli* (Asnis *et al.*, 1956), *Micrococcus denitrificans* (Vernon, 1956), *M. avium* (Yamamura *et al.*, 1955) and *M. phlei* (Brodie and Gray, 1957), *Neurospora crassa* (Tissieres and Mitchell, 1954), *Pseudomonas denitrificans* (Vernon, 1956) and *Pseudomonas fluorescens* (Stanier *et al.*, 1953), *Rhodospirillum rubrum* (Vernon and Kamen, 1954; Woody and Lindstrom, 1955), *Sarcina lutea* (Smith, 1954*b*), and *Streptomyces fradiae* (Birk *et al.*, 1957). This correlation, of course, gives no direct information about the role of cytochrome *b* in the succinoxidase systems of microorganisms. Cytochromes *c* or $c_4 + c_5$, a_1, a_2, or $a + a_3$, and apparent cytochrome oxidase activity may or may not be present, depending on the organism.

The complete absence of any kind of cytochrome *c*, cytochrome oxidase activity, or effect of mammalian cytochrome *c* on the succinoxidase system indicate clearly that neither a *c*-type cytochrome nor a conventional cytochrome oxidase is involved in the succinoxidase system of *P. tularensis*. Also absent is the antimycin A-sensitive cytochrome *c*-reducing component of mammalian systems, as indicated by the insensitivity of this oxidase to antimycin A, ascorbate, and cysteine. Similar resistance to antimycin A occurs in the succinoxidase of *A. agilis* (Repaske, 1954), and in bacterial reduced diphosphopyridine nucleotide oxidases in general.

Inhibition of the oxidase by cyanide and sulfide implies the presence of a metal-containing component in addition to cytochrome b_1 in the system, possibly the cytochrome a_1, or a_2, or both. As with the succinoxidases of *A. agilis* (Repaske, 1954), *C. diphtheriae* (Pappenheimer and Hendee, 1947) and *S. aertrycke* (Kun and Abood, 1949), this oxidase requires relatively high concentrations of cyanide for inhibition.

The effect of sulfide on *B. subtilis* is another example of environmental modification of the cytochrome composition of microorganisms. Smith (1954*b*) and Clark *et al.* (1955) have discussed the effects of oxygen supply and iron content of the medium on cytochrome composition. It is interesting that Chaix and Kuin (1942) reported that hydrogen sulfide did not inhibit the reoxidation of the cytochromes of *B. subtilis*.

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SUMMARY

The succinoxidase system of *Pasteurella tularensis* is located in the particulate fraction of the cell, and is associated and interacts with cytochrome b_1. Cytochromes a_1 and a_2 are also present; no *c*-type cytochrome or cytochrome oxidase was found. Antimycin A, ascorbate, and cysteine had no effect on the succinoxidase; cyanide and sulfide inhibited it. The inhibition by sulfide, but not by cyanide, was reversed by cysteine. High levels of sulfide, in the presence of cysteine, had no effect on growth.

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