STUDIES ON THE SUCCINOXIDASE SYSTEM OF *BACTERIUM TULARENSE*

by

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SCOPE OF THE PROBLEM

In a study of the cysteine metabolism in the microorganism Bacterium tularense it was demonstrated (1) that this organism contains a desulfhydrase which breaks down cysteine to hydrogen sulfide, ammonia and pyruvic acid. This enzyme is apparently functional during the growth stages of this organism and large quantities of hydrogen sulfide accumulate in the growth media, and its immediate atmosphere, produced from the initially high cysteine. The question can be raised, "What effect does this high concentration of hydrogen sulfide have on the electron transfer mechanism in this organism?" In fact, one might raise this question in considering any organism which produces hydrogen sulfide during growth and wonder how these organisms are able to respire in the presence of a substance which is known to be highly inhibitory to certain respiratory enzymes.

In an attempt to find an answer to this question the iron containing respiratory pigments of the microorganism Bacterium tularense were investigated. The succinoxidase system was chosen for the elaboration of the respiratory mechanism in this organism.

INTRODUCTION

The fundamental life processes for all organisms, be it the simple one-celled existence of the bacterial cell or complex dynamic interaction of the mammalian body, dwell in the energy exchange between the organism and its environment. For most, if not all of the organisms, this energy exchange is achieved by biological oxidation processes in which some substance is oxidized to yield its energy in a form useful to the organism. The oxidation-reduction reactions which have been tapped for energy vary through the families and species of the microorganisms. A basis for the classification of bacteria is in use wherein those organisms utilizing the oxidation of organic compounds, called heterotrophs, are distinguished from those oxidizing simple inorganic compounds, called autotrophs. In some bacteria one finds oxidation-reduction reactions in which oxygen participates directly in the oxidation process as the terminal electron acceptor; these bacteria are dependent on the presence of oxygen in their environment for maintainence of life. Other microorganisms carry out oxidations in which oxygen plays no role, but rather the energy is tapped by substrate dissimilation reactions whereby a complex substrate is metabolized to two or more simple products having lower free energy content than the substrate. The difference in available energy content between the substrate and

its products represents the net energy gain achieved by the organism in carrying out the metabolic reaction.

The enzymatic mechanisms by which oxidation-reduction reactions are carried out have been problems to which a great deal of effort has been applied. By far the most studied are those involving the oxidation of a substrate with the concomitant reduction of oxygen.

The earliest contribution to the problem of biological aerobic oxidation was that of C. A. MacMunnin the year 1886. (2) From spectroscopic observations of animal tissue he first described cytochrome absorption bands. His work went unrecognized until 1925 when Keilin (3)(4) published the first in a series of papers dealing with the occurence and behavior of the cytochrome respiratory pigments in plant, animal and bacterial tissues. These publications led the way toward the enlargement of our knowledge of biological oxidation, particularly the role of the cytochromes as the enzymatic agents which transfer electrons from the dehydrogenase to oxygen.

The cytochromes are ubiquitous enzymes, found in all forms of life which utilize oxygen. Thus one may correlate anaerobiosis with a lack of cytochromes in the cells of the organism. All members of the Clostridia, which are designated as strict anaerobes, contain no cytochromes. On the other hand, one finds one or more cytochrome pigments in all those organisms which are classified as facultative

or obligate aerobes.

The cytochromes have been shown to be (3)(4)(5)(6)(7) iron porphyrin enzymes which carry out electron transfer from dehydrogenase to oxygen. They show no absorption band in the visible spectrum when in the oxidized form, but when treated with the proper substrate - dehydrogenase system or with the proper chemical reductant they show three intense primary absorption bands in the visible region of the spectrum. These primary absorption bands are caused by compounds called cytochromes a, b, c. Cytochrome a is that complex of cytochromes having absorption spectra at 590-610 mu. in the red region and containing at least three cytochromes. Cytochrome a and a_1 are believed non autooxidizable, and cytochrome a_3 has been identified as the respiratory enzyme cytochrome oxidase, which is autooxidizable.

Cytochrome b is also a complex of cytochromes having at least two members, which have absorption spectra at 560 - 565 mu. and which appear to be autooxidizable.

Cytochrome c is the only cytochrome isolated in pure form. It differs from all the others in that it is a soluble, thermostable, relatively small molecular weight enzyme having an absorption spectrum at 550 - 555 mu. It is not autooxidizable.

By the use of oxidation-reduction dyes, the O-R potentials for the cytochromes have been determined (8). This knowledge, together

with that accumulated by the use of selective inhibitors has served as foundation for a theory propounded mostly by Keilin, Slater and Warburg, concerning the mechanism of electron transfer to explain biologic oxidations involving oxygen. It is held that electron transfer takes place from the dehydrogenase coenzyme (DPN or Flavin) to cytochrome b, which has the lowest O-R value of the cytochromes. The reduced cytochrome b then may transfer electrons to cytochrome c, which has the next highest O-R potential value with a similar valence change in the iron of this porphyrin enzyme. Similarly the reduced cytochrome c may reduce the cytochrome a which in turn reduces cytochrome oxidase or cytochrome a_3. The latter enzyme is rapidly re-oxidized by molecular oxygen producing one mole of water for a two electron transfer.

Probably the most studied system of which the cytochrome oxidation mechanism is a part is the succinoxidase system. This system, which is responsible for the oxidation of succinic acid to fumaric acid, has been shown to consist of a dehydrogenase, cytochrome b, an unknown factor (the Slater factor), cytochrome c, and cytochromes a, a_3. In 1909 Thunberg (9)(10) discovered an enzyme system which catalyzes the oxidation of succinic acid. His finding was corroborated by the independent observations of Batelli and Stern, (11). The importance of this system was first recognized by Szent-Gyorgyi (12) who advanced a theory of biological oxidation which involved the

mediation of hydrogen transport by the succinate-fumarate system between the majority of oxidizable metabolites and the cytochrome system. At the time this theory was first advanced, succinic dehydrogenase was the only system definitely shown to reduce the cytochromes. Considerable evidence that this mechanism (so called dicarboxylic acid cycle) is operative in normal tissues, has been marshalled by Szent-Gyorgyi and his school. It could be demonstrated that in normal tissues there is sufficient cytochrome-cytochrome oxidase and succinic dehydrogenase to account for all of the respiration through succinate-fumarate. Also, from the known O-R potential of succinate-fumarate, which lies at 0.0 volts, this system seemed to hold a key position between DPN, the flavoproteins, other metabolites and the cytochrome respiratory enzymes. Notwithstanding all the arguments mobilized and the experimental evidence brought forth, this concept has been challenged by a number of workers who hold evidence which in effect, does not destroy the Szent-Gyorgyi theory, but rather indicates that other oxidation systems can reduce the cytochromes. These workers therefore conclude that other systems can carry out the biological oxidation of a substrate not involving the succinate-fumarate system. The discovery of the flavo protein enzymes, which mediate oxidation of the reduced co-enzymes also argue against the Szent-Gyorgyi theory. The cytochrome c-reductase of yeast is capable of

reducing cytochrome c without the intervention of a succinate-fumarate system; effectively by-passing the necessity of a succinic dehydrogenase electron transfer mechanism. It thus seems likely that similar enzyme catalysts exist in animal tissue which need not involve the intermediation of the succinate-fumarate system.

Nevertheless, the importance of succinic dehydrogenase may be assumed if only for its ubiquitous occurence in the cells of most tissue.

In 1940 Keilin and Hartree (13) published an extremely lucid account of a series of experiments which they carried out on the succinic dehydrogenase-cytochrome system of cells. Pointing out that the succinic dehydrogenase system in the cell free, colloidal preparation behaves like the respiratory system of the intact cell, showing high catalytic activity and being affected by all inhibitors in the same way and to the same degree as the intact cells, they sought to consider the individual enzymes concerned with this system. They showed that the addition of soluble cytochrome c to washed heart or kidney preparations produced a marked increase in the rate of oxidation of succinic acid. Methylene blue addition also increased this rate of oxidation but not as markedly. The inhibition of the succinoxidase preparation by potassium cyanide could be reversed by the addition of methylene blue.

They were able to determine the turn-over number of cytochrome c in their heart preparation and in living yeast cells. By adjusting the concentration of the heart muscle preparation and of cytochrome c so as to obtain a maximum increase in the oxygen uptake with the minimum amount of cytochrome c added, they showed that the turn-over number of cytochrome c, which is the ratio of oxygen uptake per minute to the oxygen equivalent of cytochrome c added, was about 1420. For the determination of the estimated turn-over number of cytochrome c in living yeast cells the intensity of the alpha band of cytochrome c in the yeast suspension was matched spectroscopically with that of a standard solution of pure cytochrome c. In this case the turn-over number of cytochrome c was equal to 3850.

Among the inhibitors studied by these authors were potassium ethyl xanthate, sodium tetrathionate, and potassium persulfate. All three substances inhibited the succinoxidase system in rather small concentrations. The authors assumed that the inhibition of succinic dehydrogenase by these substances was probably similar to that produced by oxidized glutathione, which had been previously demonstrated by Hopkins and Morgan (14). The mechansim of this inhibition consisted of the oxidation by oxidized glutathione of the SH groups which are present in the enzyme and which are essential to normal activity. They also concluded that the integrity of the SH groups

is essential for the normal activity of succinic dehydrogenase and that these groups do not undergo reversible oxidation during the catalytic oxidation of succinic acid.

From the O-R potential of cytochrome b Keilin and Hartree postulated a role for cytochrome b in the succinic acid oxidase. Slater (15) proposed a scheme for the succinic oxidation system which included cytochrome b as an intermediate between succinic dehydrogenase and oxygen.

In 1947 Pappenheimer and Hendie (16) studied the relationship between the iron enzymes of Corynebacterium diphtheriae and diphtheria toxin. They obtained evidence that cytochrome b was directly concerned with the oxidation of succinic acid. The oxygen uptake of the sonic extracts to the diphtheria bacillus on succinate was proportional to the amount of iron added to the media from which the organisms were cropped. Parallel to this was their finding that the intensity of the absorption band at 560 mu. of their sonic extracts was greater in organisms grown on a high iron media in contrast to those obtained by growth on a low iron media. When succinate was added to diphtherial extracts under anaerobic conditions, the bands of reduced cytochrome b quickly became visible with a hand spectroscope. When air was introduced into the Thunburg vessel the intensity of the absorption band at 560 mu. was reduced. Further, diphtherial cytochrome b appeared to be associated with the large

molecular components of the diphtherial extracts with which the succinoxidase activity was also associated. After a long period of centrifugation in the ultracentrifuge, 70% of the cytochrome b and 70% of the succinoxidase activity were found in the sedimented material.

In another paper, Pappenheimer and Hendie (17) compared the diphtherial succinoxidase system with that of beef heart muscle. They demonstrated that the primary differences between mammalian and bacterial succinoxidase systems resided in the varying amounts of cytochrome components present. Thus the beef heart muscle contained relatively large amounts of cytochrome c, cytochrome oxidase, and the factor (Slater factor) which mediates the reduction of cytochrome c by cytochrome b. The diphtheria bacillus possessed far more succinic dehydrogenase activity and cytochrome b, but only traces of cytochrome c and cytochrome oxidase. The authors concluded that the rate limiting step in the succinoxidase system was not the initial oxidation of succinate to fumarate but rather a step in the re-oxidation of the cytochrome b. In the presence of methylene blue or ferricyanide the rate of succinate oxidation was increased 25-30 fold. Since the succinic acid dehydrogenase activity measured by the methylene blue or the ferricyanide method was always directly proportional to the cytochrome b content, the authors further concluded that the succinic dehydrogenase is a hemin containing

enzyme which may be identical with cytochrome b.

The conclusions of Pappenheimer and Hendie were challenged by Rawlinson and Hale (18). In their communication they pointed out that the microorganism Corynebacterium diphtheriae showed primarily alpha bands at 601 and 562 mu. and a beta band at 531 mu. corresponding to cytochrome a_3 and cytochrome b. They concluded that the main haem compounds are cytochrome b and cytochrome oxidase. Further they isolated the hemochromogens which correspond to the prosthetic group of cytochrome oxidase and were able to differentiate this from cytochrome a.

The identity of cytochrome b and succinic dehydrogenase was further emphasized by the examination of the O-R potential of the two systems. Ball (19) pointed out that if cytochrome b functions in the respiratory enzyme chain the possibility seems rather small that another system lies between it and succinoxidase or the flavo proteins. Moreover, the increment of the total energy released in the oxidation of the proposed dehydrogenase by cytochrome b, would be negligible.

It had generally been considered that unlike the oxidases and peroxidases the dehydrogenases were insensitive to cyanide. Evidence was brought forth by Dixon and Keilin (20) that xanthine oxidase, which is a flavin enzyme, is slowly but irreversibly inactivated by cyanide. This was the first example of cyanide sensitivity of a

dehydrogenase. Unlike the reversible inhibition of cytochrome oxidase by cyanide, the xanthine oxidase was irreversibly inhibited. Tsou (21) investigated the effects of cyanide on succinic dehydrogenase and concluded on the basis of his studies that succinic dehydrogenase was not identical with cytochrome b. He demonstrated that succinic dehydrogenase was slowly and irreversibly inactivated by cyanide concentrations of the order of 5×10^{-3} M. After 24 hours at room temperature the activity of the dehydrogenase was less than 2% of the original. On the addition of dihydrocozymase there appeared a strongly reduced cytochrome c band together with a weaker b band. Moreover, the cytochrome b present in the cyanide treated enzyme preparation could also be reduced by the addition of a few drops of an untreated enzyme followed by succinic acid although this reduction was rather slow. He argued that if the succinic dehydrogenase were identical to cytochrome b it would be possible on incubation with cyanide, to observe a parallel relation between the succinic dehydrogenase activity and the intensity of the alpha band of reduced cytochrome b. Since Tsou was unable to demonstrate any parallel relationship, he concluded that the cytochrome b was not identical to succinic dehydrogenase.

Also in clear contrast to the work of Pappenheimer and Hendie is the report of Johns (22) who studied the succinic dehydrogenase activity of a strictly anaerobic micrococcus identified as Veillonella

gazogenes. This organism showed both succinic acid and lactic acid dehydrogenase activity and, as a strictly anaerobic bacteria, did not contain any spectroscopically visible cytochrome components. If cytochrome b were essential for succinic dehydrogenase activity it is obvious that one would not expect to find dehydrogenase activity without cytochrome b. The finding of such activity in the absence of cytochrome b is suggestive of their non-identity.

With a rather similar approach Shepherd (23) studied the succinic dehydrogenase and succinic oxidase of Neurospora. He pointed out that the cytochrome system had not been demonstrated as yet in this organism, but he was able to demonstrate that cytochrome c markedly stimulated the rate of succinoxidase activity. He observed certain curious aberrations from the generally common findings in the studies of this enzyme in other tissues. The enzyme was rather unstable. It had a curious double pH optimum. The succinic dehydrogenase activity was not inhibited by 2×10^{-3} M cyanide. However, the succinoxidase system was completely inhibited by that concentration of cyanide.

Further, Kun and Abood (24) were able to demonstrate an entirely different succinoxidase system in Salmonella aertrycke. Their experiments demonstrated that the succinoxidase found in this organism could oxidize succinic acid with molecular oxygen. Kun and Abood demonstrated a similar pH activity curve as previously reported in

other tissue preparations and were also able to demonstrate the reversibility of the system. Their enzyme preparation, however, was inhibited by cytochrome c and by methylene blue. This inhibition was shown to be competitive. They had evidence that the competition occurred between cytochrome c and a part of the succinoxidase which participates in aerobic oxidation. Cytochrome c was reduced by the Salmonella enzyme but not re-oxidized. They suggested that the reduction of cytochrome c is brought about by the same component - that is, flavin - which activates molecular oxygen in the Salmonella preparation. This circumstance could explain the competitive inhibition of succinate oxidation by cytochrome c.

Attempts to demonstrate a cytochrome system in their preparations were unsuccessful. Since the extract of the Salmonella enzyme had a flavin-like spectrum and since evidence was obtained for the formation of hydrogen peroxide during aerobic succinate oxidation the authors reasoned that the reaction of molecular oxygen was mediated by a catalyst similar to a flavo protein.

Flavo-proteins have been postulated to be part of the animal succinoxidase system (25). Keilin and Hartree (13) found some evidence to support this. By means of spectroscopic analysis their experiments indicated that a flavin component participates in aerobic succinate oxidation. Experiments were designed to

demonstrate the role of flavin-succinate oxidation were carried out by Axelrod, Potter, and Elvehjem (26) who found that in riboflavin deficient rats a component of the succinoxidase system was diminished throughout the tissues of the body. This component was not succinic dehydrogenase or the cytochrome system and could be restored by riboflavin feeding.

Tsou (21) also suggested the possibility that succinic dehydrogenase was a flavin enzyme since he saw some similarity between the inactivation by cyanide of succinic dehydrogenase and the inactivation by cyanide of xanthine dehydrogenase whose prosthetic group is FAD.

Chance (27) using sensitive spectrophotometric techniques capable of recording rapid optical density changes studied the rate of succinic acid oxidation and concomitantly the rate of optical density changes of the cytochrome pigments. By comparing the per cent oxidized in the steady state to the ratio of the oxygen consumed per change in optical density unit, he concluded that cytochrome b is not the same as succinic dehydrogenase and does not play a direct role in the succinoxidase system. Chance's determinations of the rates of reduction of cytochromes c, a, and a_3 either during the exhaustion of oxygen or in the presence of cyanide are in agreement with the participation with these three cytochromes in the system. Slater (28) took exception

to Chance's conclusions that cytochrome b does not play a direct role in the succinoxidase system. He pointed out that Chance's data supported a conclusion that cytochrome b is directly involved in the system. The mathematical treatment by Chance ignored the possible back-reactions; on the basis of Ball's data (8) for the O-R potential of cytochrome b, the back-reaction, that is, the reaction of fumarate plus reduced cytochrome b could be considerable. Indeed, Ball's data suggests that only 35% of the cytochrome b would be in a reduced state at the end of Chance's experiment.

In a series of papers on the succinoxidase system in heart muscle and in kidney, Slater (14)(29)(30)(31) crystallized many of the prevailing ideas concerning the mechanism of electron transfer by this system and succeeded in uncovering a number of pertinent facts which dealt with the role and sequence of cytochrome b, an unknown factor (Slater factor), cytochrome c, cytochrome a and cytochrome oxidase. Recognizing the previous suggestions that cytochrome b and succinic dehydrogenase were identical Slater avoided making any finite statement based on his work; however, he pointed out that when succinate was introduced anaerobically to his tissue preparation, the b band and the other cytochrome bands became evident. On the addition of methylene blue the b band immediately and almost completely disappeared while the c and the a plus a_3 bands remained visible. This observation strongly suggested that

cytochrome b was involved in the reduction of methylene blue but he was unable to state whether cytochrome b was directly reduced by succinate or if its reduction required an additional enzyme which is the true succinic dehydrogenase. His finding that the activity of the succinic dehydrogenase and of the succinoxidase system were about three times as high in heart muscle as in kidney preparations and that this ratio is similar to the relative intensity of the cytochrome b band, agrees with the conclusion that cytochrome b is closely associated with succinic dehydrogenase.

The work to be described in this thesis deals with the cytochrome pigments of Bacterium tularense and the role of cytochrome b in the succinoxidase activity of Bacterium tularense. An attempt has been made to characterize cytochrome b and succinic dehydrogenase in order to throw some light on the possible identity of these two enzymes.

METHODS

A. Cultural Methods

Stock cultures of Bacterium tularense, strain Schu, were maintained on glucose cysteine blood agar (32). Liquid cultures were grown on casein hydrolysate decamin medium of the following composition: 1% sodium chloride, 2% glucose, 2% acid hydrolyzed casein (vitamin free), 0.3% Decamin*, 0.2% cysteine·hydrochloride, 0.2 gamma thiamine/ml., 0.0225 M potassium phosphate, 0.0002 M magnesium sulfate, 0.0004 M calcium chloride, 2×10^{-7} M ferrous sulfate, and 2×10^{-7} M manganese sulfate. The medium was adjusted to pH 6.5 with potassium hydroxide and autoclaved at 15 lbs. per square inch at 121° C. for 12 minutes.

Whole cells were obtained by the inoculation of 400 ml. of liquid medium with a heavy inoculum obtained from 24 hour growth on liquid medium. The medium was incubated 12-18 hours at 37° C. with shaking. At the end of this period of time the cells were centrifuged, washed with 0.9% sodium chloride solution, centrifuged and finally suspended in 20 ml. of 0.1 M phosphate buffer pH 7.4. Approximately 5 gms. of organisms wet weight were obtained.

Cell free extracts were prepared from this suspension. The 20 ml. of suspended cells were placed in the chamber of the Raytheon

* Autolysed yeast. Vico Products Company, Chicago, Illinois.

sonic disintegrator and maintained at a temperature below 10° C. by passing ice water through the cooling chamber. The cells were treated for a period of 30 minutes with 100 plate volts at 9000 cycles - this being sufficient to disintegrate 99% of the cells. The resulting suspension was centrifuged for 30 minutes at 3000 x g to remove the small amount of debris. The final supernate was practically free of cells and contained 4 mg. nitrogen per ml.

B. Respiration Experiments

Oxygen consumption was measured in the Warburg respirometer at 37° C. according to standard procedures. The gas phase was air. The final volume was 3 ml.

C. Preparation of Washed Particles

20 ml. of the sonic extract obtained as above were centrifuged in the Spinco ultra centrifuge for 3 hours at 105,000 x g. At the end of this period of time the supernatant was decanted off the reddish precipitate in the bottom. This precipitate was taken up in 20 ml. of 0.1 M phosphate buffer and centrifuged for 1 hour in the high speed head of the International centrifuge at 20,000 x g. The supernatant was discarded, the precipitate once again taken up in buffer and centrifuged. The precipitate finally obtained was called "washed particles."

D. The Synthesis of Ethyl Hydrogen Peroxide (33)(34)(35)

To a flask containing a mixture of 170 gm. of 10.8% hydrogen

peroxide and 177 gm. of 42% potassium hydroxide was added with stirring, 100 gm. of diethyl sulfate (EK white label) until the latter disappeared. The whole reaction mixture was cooled to 10° C. in an ice water bath during this procedure. The stirring was continued for 6 hours and then the whole mixture was acidified with dilute sulfuric acid. The entire reaction mixture was then distilled in an oil bath. The temperature of the oil bath was maintained at 105° C., the temperature of the distillation flask at 100° C. and the vapor temperature at 93-95°C. The fraction which distilled at 95° C. was collected. This fraction, which contained the ethyl hydrogen peroxide, and whose volume was 200 cc., was titrated with potassium iodide and sodium thiosulfate and found to be 2.1 N. This solution, which was impure, was purified in the following way. The solution was extracted two times with 200 cc. of ethyl ether. The ether extract was dried with sodium sulfate, filtered and the ether was stripped off under vacuum. The residue obtained was placed in a small distilling flask and the fraction that distilled at 41-42° C. at 65 mm. mercury was collected.*

* During both distillations the temperature in the flask was not allowed to go beyond 120° C. since ethyl hydrogen peroxide is violently explosive above this temperature. When the final distillation was made the oil bath used was maintained at a temperature of 60° and a precautionary mask was worn to protect in case of any explosion. The latter distillation is probably the more critical of the two. Also it is worth noting that the ethyl hydrogen peroxide is an extremely strong oxidizing agent and that contact with the skin causes severe burns.

RESULTS

A. Determination of Cytochrome Oxidase

Since it has been established that cytochrome oxidase is the respiratory pigment sensitive to hydrogen sulfide (3) it was considered useful to establish the presence or absence of this enzyme in cells and sonic extracts of Bacterium tularense.

The determination of cytochrome oxidase (or indophenol oxidase as it is called in the older literature) was initially carried out using the method of Keilin (3). This method depends on the appearance of a violet color when p-phenylenediamine or the Nadi reagent is allowed to react with tissue containing cytochrome oxidase. The experiment was carried out using fresh yeast as a control organism to demonstrate the effect, along with Bacterium tularense organisms for the determination of the cytochrome oxidase activity. In the experiment with the Bacterium tularense organisms 2 ml. aliquots of washed cells containing 12.8 mg. of nitrogen per 2 cc were added to tubes containing 1 ml. of 0.1% solution of neutralized p-phenylenediamine or 1 ml. of the Nadi Reagent.* Either reagent provided excellent results with yeast. Also included in some of the tubes were 0.001 M cyanide, in other tubes

* The Nadi Reagent is prepared fresh using equal parts of 0.01 M p-phenylenediamine hydrochloride, 0.01 M alpha naphthol in 50% alcohol, and 0.25 M sodium carbonate solution.

the organism was boiled to destroy activity. Table I shows the results of the effort to demonstrate cytochrome oxidase activity in Bacterium tularense and in yeast cells. It may be seen that the violet color was produced in only that tube containing the yeast, and that this oxidation was inhibited by boiling the organisms or by the addition of 0.001 M cyanide. These initial findings strongly suggested that cytochrome oxidase activity was absent in Bacterium tularense cells.

In another attempt, the presence of cytochrome oxidase activity was determined by the method of Cooperstein et al (36). The method depends upon the reoxidation of reduced cytochrome c by homogenate and whole tissue. The reoxidation of cytochrome c was followed in a Beckman DU spectrophotometer at a wave length of 550 mu.

The reduced cytochrome c was prepared by taking 30 ml. of a solution of cytochrome c (1.7×10^{-5} M) in 0.03 M phosphate buffer, pH 7.4, and reducing this with 100 micro-liters of a freshly prepared solution of sodium hydrosulfite (1.2 M). The solution was shaken in air for 2-4 minutes to remove the excess hydrosulfite. 3 ml. of the reduced cytochrome c solution was pipetted into a cuvette and 0.05 ml. of the tissue to be examined was added. The cuvette was rapidly inverted to insure adequate mixing and readings in optical density units were taken every 3 minutes. At the end of

Table I

The Absence of Cytochrome Oxidase in

Bacterium tularense

	Activity**
Whole cells*	-
Whole cells* + potassium cyanide†	-
Whole cells* heated to 100° C. for 5 min.	-
Control Yeast cells‡	+ + + +
Yeast cells‡ + potassium cyanide†	-
Yeast cells‡ heated to 100° C. for 5 min.	-

** The appearance of the violet color in 10 min. indicated activity.

* *Bacterium tularense* whole washed cells 6.4 mg. N/cc; 2 cc/vessel.

† Potassium cyanide 1 x 10^{-3} M final conc.

‡ Bakers yeast washed cells 5 mg. N/cc; 2 cc/vessel. Each vessel contained 2 cc. of cells; 1 ml. of Nadi reagent. 2 ml. of 0.1 M phosphate buffer pH 7.0 plus addition indicated to final volume of 5 ml.

12 minutes a crystal of ferricyanide was added to oxidize the cytochrome c and the optical density of this solution was determined. In Figure I it is apparent that whole Bacterium tularense organisms and sonic extracts did not reoxidize the reduced cytochrome c, while a control of rat liver homogenate very rapidly reoxidized the reduced cytochrome c. For this experiment whole cells and sonic extracts were obtained as described under Methods. Both preparations contained 4 mg. nitrogen/ml.; 0.04 ml. of either whole organisms or sonic extract were used. For the rat liver control, a rat was anesthesized with chloroform and the liver rapidly removed and washed with distilled water. The whole liver was placed in a Waring Blender with 100 ml. of ice cold 0.3 M phosphate buffer, pH 7.4, and homogenized for 5 minutes. The homogenate was diluted 1:300 and 1:30,000 with the same molar concentration of phosphate buffer.

B. The Reduced Absorption Spectra of Bacterium tularense

(1) Spectrographic Studies

Using a Bausch and Lomb medium quartz spectrograph, the reduced absorption spectra of Bacterium tularense was determined. For this purpose cells were grown as described in the Methods section and the packed cells were suspended in an equal volume of 0.9% saline. This heavy cell paste was placed in a Bausch and Lomb cell having a light path of 10 mm. after the addition of a few grains

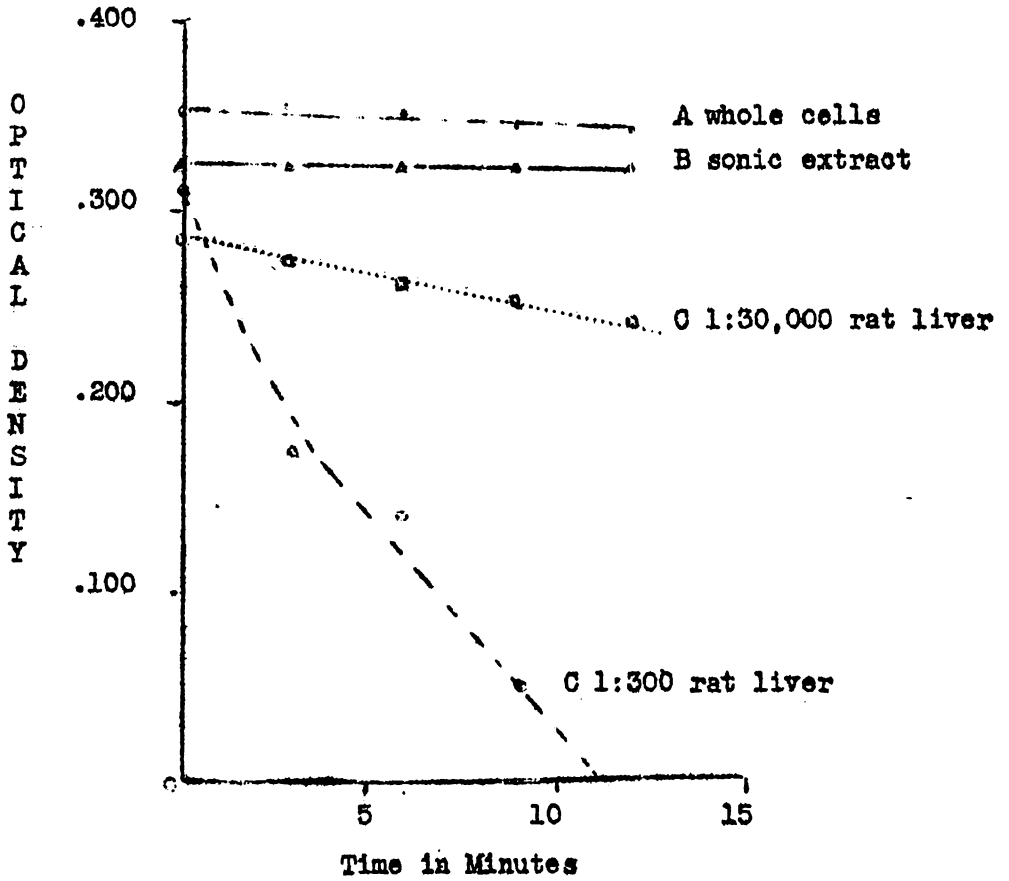

Fig. I. The Determination of Cytochrome Oxidase in B. tularense. Each cuvette contained 3.0 ml. of reduced cytochrome c. At zero time 0.05 ml. of either A, whole cells, B, sonic extract, or C, homogenate was added as indicated. A control cuvette contained reduced cytochrome c without any additions. The blank was 0.03 M phosphate buffer.

Optical density readings were determined at 550 mu. with a Beckman DU spectrophotometer at 3 minute intervals.

of sodium hydrosulfite. By changing the slit width and exposure time of the panchromatic spectrograph plates the proper conditions were arrived at to demonstrate the presence of a band at 550 and a very weak band at 530 mu. No other bands were in evidence. As a control the absorption bands of yeast cells were determined and all three alpha bands of cytochromes a, b, c plus a diffuse d band was evident.

(2) Difference Spectra

The difference spectrum of sonic extracts of Bacterium tularense was determined using the Beckman DU spectrophotometer. The blank contained 3 ml. of the sonic extract. The unknown contained 3 ml. of the sonic extract plus a few crystals of sodium hydrosulfite. Figure II shows this difference spectrum. The alpha peak is at 560 mu., the beta at 525 mu., and the gamma peak or Soret band is at 432 mu. No other peaks are in evidence. An essentially identical difference spectrum was obtained using sodium desoxycholate at a final concentration of 2%. See Figure III. The advantage in the sodium desoxycholate procedure is evident by comparing Figures II and III in that the absorption peaks are better resolved and more clearly evident at the lower wave lengths.

(3) Spectroscope

For the routine examination of the absorption spectrum of sonic extracts the hand spectroscope was found to be the most useful.

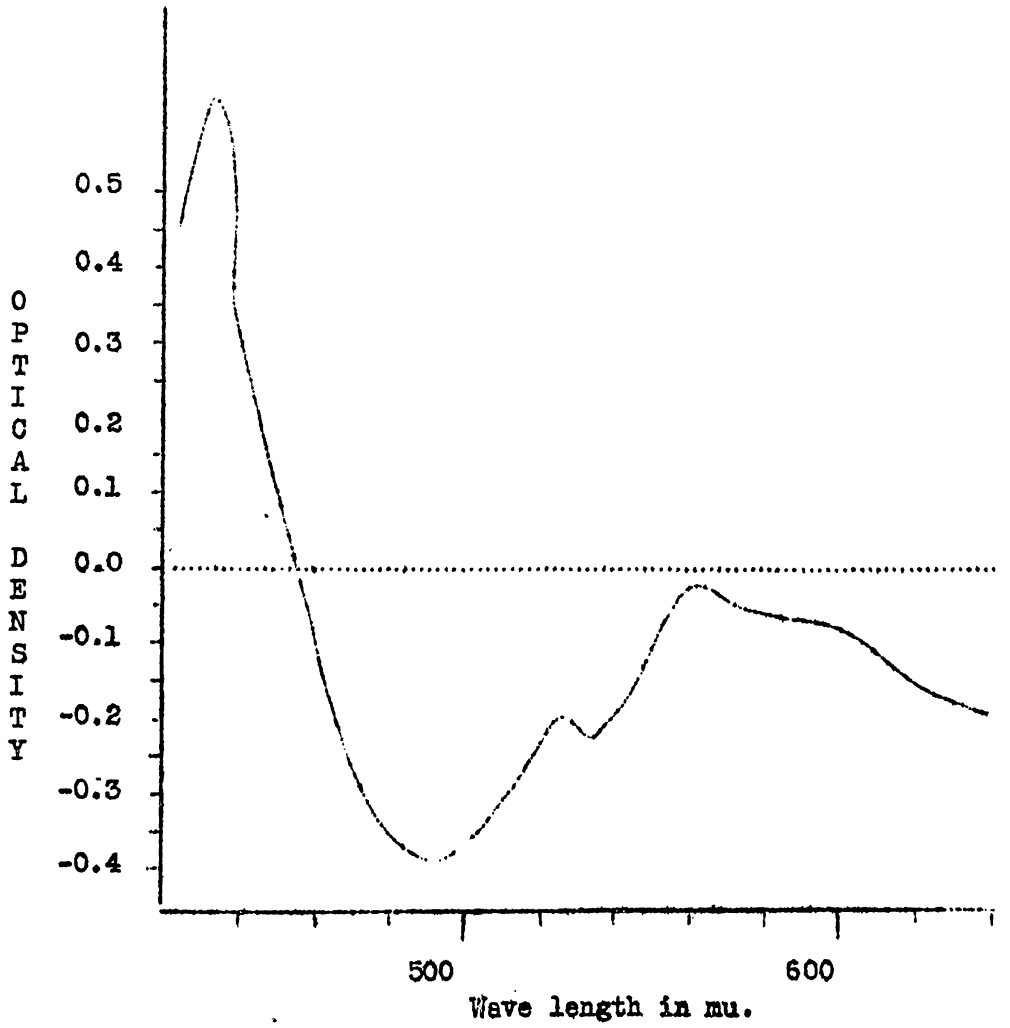

Fig. II. The Difference Spectrum of Sonic Extracts of B. tularense
A blank cuvette containing 3.0 ml. of untreated sonic extract was matched against a cuvette containing sonic extract plus a few crystals of sodium hydrosulfite.

Optical density was determined at 5 mu wave length intervals with a Beckman DU spectrophotometer.

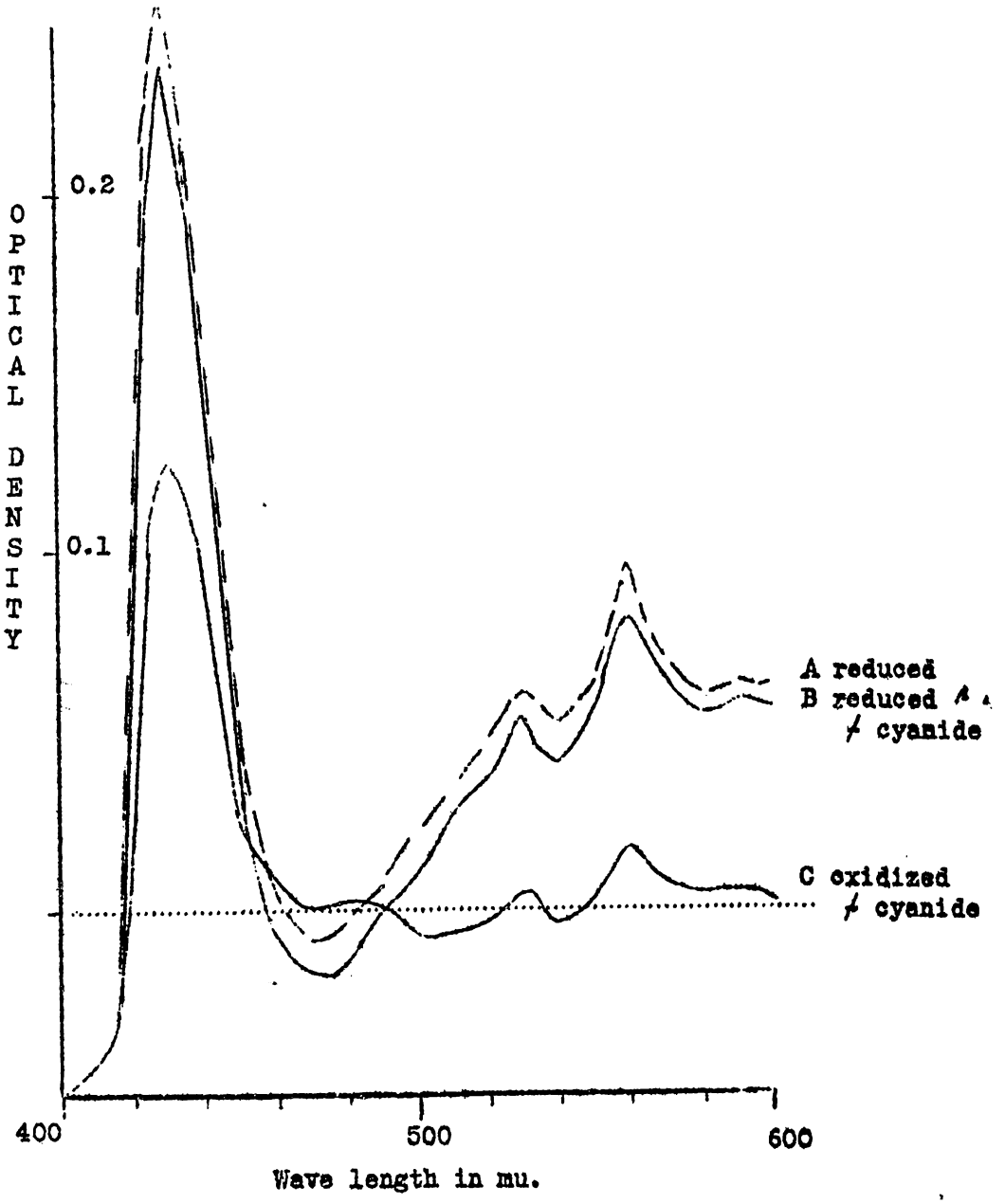

Fig. III. The Difference Spectra of Desoxycholate Treated Sonic Extract with and without Cyanide. A blank cuvette containing 2.9 ml. sonic extract (treated with sodium desoxycholate, 2% final concentration) plus 0.1 ml. 0.1 M phosphate buffer pH 7.4, was matched against: A. a cuvette containing the treated sonic extract + a few crystals of sodium hydrosulfite, B. a cuvette identical to A but containing 4×10^{-3} cyanide, and C. a cuvette containing treated sonic extract + 4×10^{-3} cyanide without sodium hydrosulfite. Optical density was determined at 5 mu wave length intervals with a Beckman DU spectrophotometer.

Whole cells or sonic extracts treated with a few crystals of sodium hydrosulfite showed a strong band at 560 mu. and a weak band at about 525 mu. It should be noted here that a weak band at about 635 mu. was evident with a hand spectroscope, viewed with a light passing through at least 5 cm. of the sonic extract. It was assumed that this band was the catalase band since it did not require reduction with a reducing agent to manifest its presence and it was so weak that it could only be observed through extremely thick solution.

C. The Absence of Cytochrome b in Bacterium tularense

From an examination of the absorption peaks in sonic extracts of Bacterium tularense the conclusion might be reached that the only cytochrome present in this organism is cytochrome b. The sharpness and symmetry of the peak in the Soret region helps to strengthen this contention. (See Fig. III). Further, examination of sonic extracts with a hand spectroscope at room temperature and at the temperature of liquid air did not resolve the absorption peak at 560 mu. into any more bands. When these sonic extracts were treated at a temperature of 80° C for 20 minutes and a reducing agent was added all bands disappeared. Since cytochrome c is heat stable, examination with a hand spectroscope would permit its observation. When beef cytochrome c was added in small amounts (4×10^{-7}M) to sonic extracts the two bands for c and b could

easily be seen. Heat treatment of this solution destroyed the cytochrome b but allowed observation of the cytochrome c.

Finally an attempt to express cytochrome c from Bacterium tularense was carried out. Using the method of Keilin (4) for the extraction of cytochrome c from yeast, no cytochrome c was obtained from Bacterium tularense. A parallel experiment rum with Bakers yeast was performed and easily yielded the cytochrome c. For the determination with Bacterium tularense 10 gm. of organisms wet weight were collected, heated to a temperature of 90° C. and cooled by addition to ice. The solution was allowed to stand 4 hours, centrifuged and the cake of organisms was treated with 1/2 gm. of sodium bisulfite and 1/2 gm. of sodium sulfite. The solution was allowed to stand overnight in the ice box, centrifuged and the absorption spectrum of the clear liquid was determined in the Beckman B spectrophotometer. No absorption bands were in evidence. With the yeast cake the alpha band at 550 was evident as well as the Soret band at 415. From these experiments one may conclude that no cytochrome c may be found in Bacterium tularense.

D. The Relationship of Succinoxidase to Cytochrome b.

When a solution of succinate was added to sonic extracts of Bacterium tularense in a Thunburg tube and evacuated with a water pump, the absorption band of cytochrome b at 560 mu. was apparent when examined with a hand spectroscope. After opening the tube to

air, the band at 560 mu. immediately diminished in intensity but did not disappear.

In a typical experiment, 1.5 ml. of the sonic extract was placed in the main compartment of a Thunburg tube. In the side arm was placed 1.5 ml. of a 0.1 M solution of succinic acid, pH 7.2. The tube was evacuated with a water pump and examined with a hand spectroscope. Before the addition of the succinic acid no absorption band was evident. Immediately on tipping the succinate into the sonic extract, a band appeared at 560 mu. Admitting air to the vessel caused a diminution in the absorption intensity but it did not completely reoxidize. The diminution in the intensity of the absorption band indicated autooxidation of the cytochrome b. That a relationship exists between succinic dehydrogenase and cytochrome b is reinforced by the finding that the activity of the succinic dehydrogenase was associated with a heavy particulate material which could be centrifuged out of sonic extracts using high speed centrifugation. To illustrate this relationship, 20 ml. of sonic extract was centrifuged for 4 hours using an International refrigerated centrifuge with high speed head attachment. The centrifugation was carried out at 5° C. and a speed of 25,000 x g. At the end of the 4 hours there appeared on the bottom of the tube an opaque precipitate which appeared pink by transmitted light. Just above this precipitate was a cherry red solution which was over-

laid by a clear yellowish supernate. The two solutions--that is, the red solution and the yellow overlying supernate--were decanted into a second cup and centrifuged for 7 hours at the same speed and at the same temperature in the same centrifuge. After this period of time the red solution was concentrated on the bottom of the tube with the yellow solution overlying it. The red solution was withdrawn from the tube with the use of a pipette whose tip had been drawn to a fine point. This effected good separation of the two solutions. It was immediately established with the hand spectroscope that the red solution contained most of the cytochrome b. The remaining cytochrome b could be found in the precipitate material obtained initially by centrifugation. When the material obtained by centrifugation was examined for succinoxidase activity, it was clear that most of the activity was associated with the red solution, that is, the cytochrome b fraction. Table II shows the oxygen uptake obtained in this experiment. Very little succinoxidase activity was found in the yellow supernate fraction and by examination of this fraction with the hand spectroscope it was found to contain no evident band at 560 mu. The initial precipitate obtained and the cherry red solution both contained cytochrome b and both showed succinoxidase activity. The red solution having the most intense band at 560 mu. showed the greatest succinoxidase activity.

Table II

The Relationship of Cytochrome b

to Succinoxidase Activity

Enzyme Material	Cytochrome b*	Q_{O_2}**
Sonic Extract	+ +	139
"Yellow supernatent"	-	24
"Red supernatent"	+ + + + + +	544
Precipitate	+ + +	238

* Cytochrome b estimated with hand spectroscope.

** ul O_2/mg N/hr.
Each vessel contained 0.5 ml. of the material mentioned plus 100 uM of succinic acid pH 7.4 + 1.5 ml. phosphate buffer pH 7.4 to a volume of 3 ml.
Oxygen uptake measurements were based on the first 30 minutes. The center well contained 0.2 ml. water; the temperature was 37° C.

E. The Reduction of Cytochrome c by Cytochrome b in the Presence of Succinate

When purified bacterial cytochrome b (cherry red solution obtained by high speed centrifugation) or a sonic extract of Bacterium tularense was added to a solution of succinate and cytochrome c in excess the cytochrome c was slowly reduced. The reduction could be followed at 550 mu. in a Beckman DU spectrophotometer as shown in Fig. IV. Cyanide at a concentration of 1×10^{-3} M inhibited the rate of reduction of cytochrome c by cytochrome b.

F. Some Properties of Bacterial Cytochrome b

(1) Heat Stability

The cytochrome b found in sonic extracts of Bacterium tularense is a thermolabile substance. When sonic extracts of Bacterium tularense were placed in an 80° C. water bath for a period of 20 minutes the extract appeared to be coagulated. Examination for the reduced band at 560 mu. was unsuccessful using succinate or dithionate as reducing agents. Examination of the material for succinoxidase activity was also unsuccessful. We may conclude that the cytochrome b is thermolabile and parallels the thermolability of the succinoxidase.

(2) The O-R Potential of Cytochrome b in Sonic Extracts of Bacterium tularense

Using the method of Ball (3) for the determination of the O-R

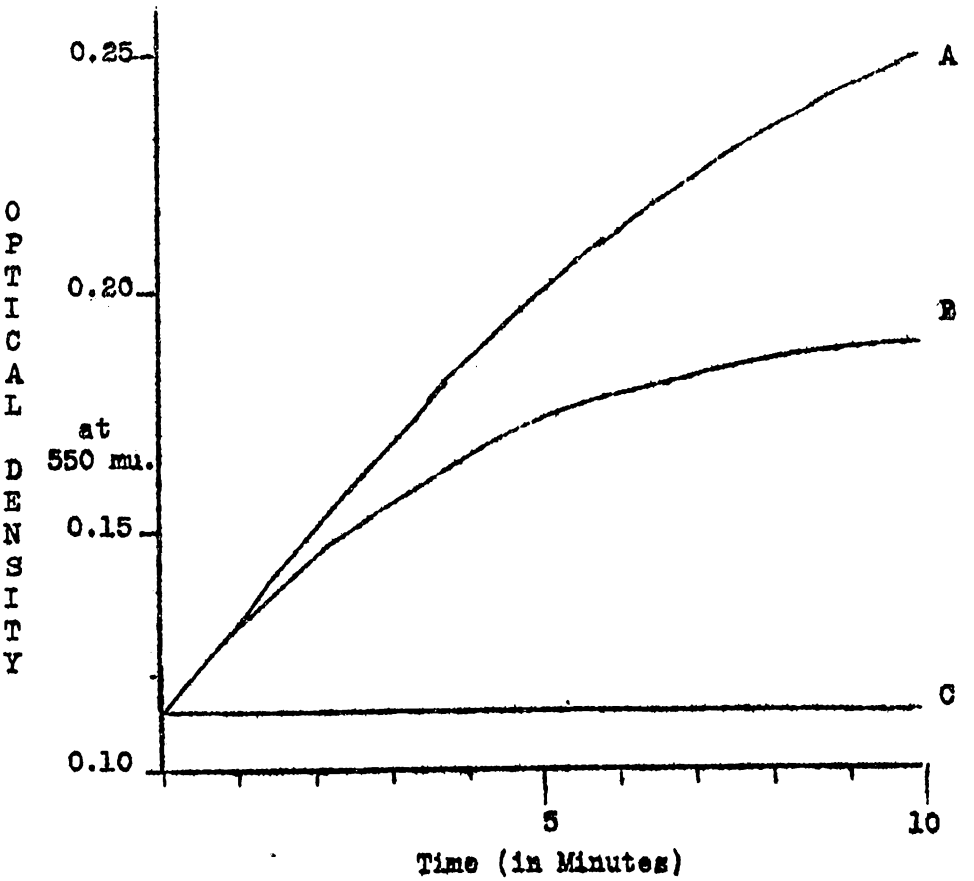

Fig. IV. The Effect of Cyanide on the Reduction of Cytochrome c by Sonic Extracts in the Presence of Succinate. Each cuvette contained 2.3 ml. 1.7×10^{-5} M cytochrome c, 0.5 ml. 0.06 M succinate pH 7.4, 0.1 ml. sonic extract, and in cuvette A 0.1 ml. phosphate buffer pH 7.4, in cuvette B 0.1 ml. 3×10^{-2} M cyanide (final concentration in cuvette was 1×10^{-3} M). The blank contained water substituted for cytochrome c. A control cuvette C contained cytochrome c, sonic extract and phosphate buffer, with no substrate added. Optical density readings were determined at 550 mu. with a Beckman DU spectrophotometer at 1 minute intervals.

potentials of the cytochromes, the O-R potential of cytochrome b in sonic extracts of Bacterium tularense was determined. When ascorbic acid was used as the reductant and the sonic extracts were examined for the reduced b band, only 5-10% of the intensity of the reduced band is evident. This was compared with the complete reduction produced by sodium hydrosulfite. From the known O-R potential of ascorbic acid the O-R potential of cytochrome b in sonic extracts of Bacterium tularense was estimated to be approximately -0.02 volts.

When cysteine was used as the reductant the cytochrome b appeared to be completely reduced. Since cysteine has such a low O-R potential, bringing about complete reduction of the cytochrome b, the O-R potential of the cytochrome b can only be approximated, and its value has been estimated to be greater than -0.08 volts. Using succinic acid as the reductant the cytochrome b band appeared to be 25% reduced. From the O-R potential of the succinate fumarate system, the O-R potential of cytochrome b was calculated to be -0.044.

(3) The Formation of a Compound by Bacterial Cytochrome b with Cyanide

When sonic extracts of Bacterium tularense were treated with 2% sodium desoxycholate the turbidity of the solution was dispersed so that the extracts now appeared clear. These cleared extracts could be more readily examined in the Beckman DU spectrophotometer.

When these desoxycholate-treated extracts were examined in the spectrophotometer with and without the addition of 4×10^{-3} M cyanide, a shift in the oxidized spectrum due to the addition of cyanide could be observed. See Fig. V. In a similar experiment using desoxycholate-treated sonic extracts, the absorption spectra by the difference spectrum method was determined, and it was observed that the cyanide did not appear to react with the reduced form of cytochrome b but only with the oxidized form. See Fig. III. From examining these spectra one can see that cyanide reacts with the oxidized form of cytochrome b to give spectra which are similar in appearance to the reduced spectrum obtained by the addition of a strong reducing agent. That the spectra so obtained were not due to the reducing effects of cyanide was shown by the fact that the addition of potassium ferricyanide to the cuvette did not dissipate the intensity of the spectrum. However, the similarity of absorption spectra of the reduced cytochrome b and the oxidized cytochrome b plus cyanide makes one hesitant in concluding that a compound is formed.

(4) The Estimation of the Concentration of Cytochrome b in Sonic Extracts of Bacterium tularense Using the Method of Slater (15)

The amount of cytochrome b in Bacterium tularense was determined spectroscopically by comparing the intensity of the band at

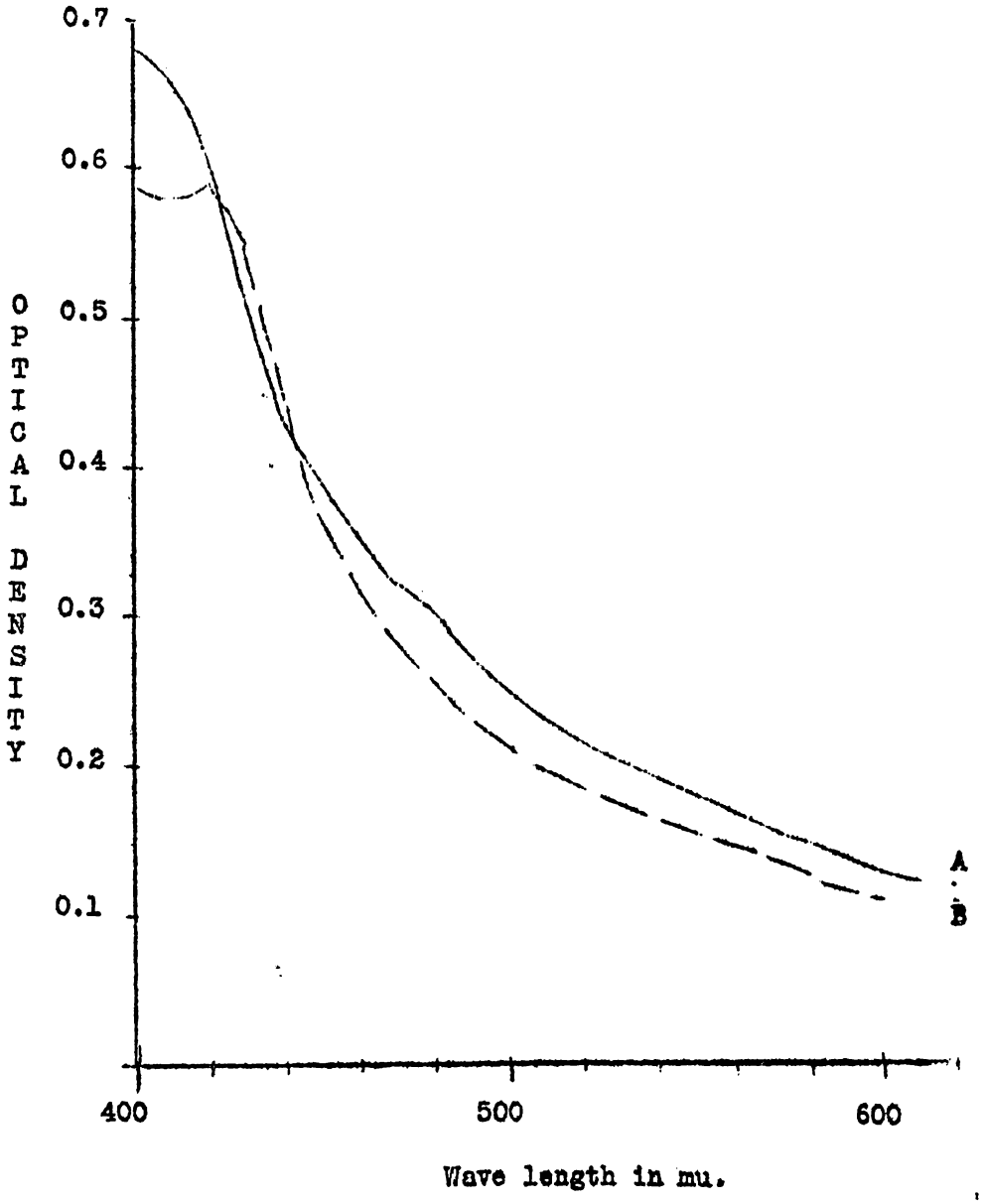

Fig. V. The Oxidized Spectra of Desoxycholate Treated Sonic Extract of Bacterium tularense with and without Cyanide. Cuvette A contained 2.9 ml. of 2% desoxycholate treated sonic extract + 0.1 ml. phosphate buffer pH 7.4. (0.1M) Cuvette B contained 2.9 ml. of the treated sonic extract + 0.1 ml. cyanide, so that the final cyanide concentration was 4×10^{-3} M. Optical density readings were determined at 5 mu wave length intervals with a Beckman DU spectrophotometer.

560 mu. with a diluted sample of cytochrome c. For this estimation the assumption was made that the extinction coefficient for the two cytochromes are approximately equal. It was estimated that the concentration of cytochrome b in sonic extracts of Bacterium tularense was approximately 4×10^{-7} M. If we assume this figure to be correct we arrive at the fact that 1 cc. of the sonic extract contains 4×10^{-4} mM. of cytochrome b. Since we have determined that the maximum rate of activity of the succinoxidase preparation is 6 uM of fumarate produced per ml of extract per minute, (see page 45), we can calculate that the turnover number of the cytochrome b is approximately 15,000.

G. Some Properties of the Succinoxidase of Bacterium tularense

(1) The Effect of pH on Succinoxidase Activity

The optimal pH for the succinoxidase activity in sonic extracts of Bacterium tularense is approximately 7.3 as seen in Fig. VI. For these determinations, the contents of the Warburg vessel which contained 100 uM of succinate in 0.1 M phosphate buffer were adjusted to the desired pH, and the oxygen uptake determined. The microliters of oxygen uptake in 50 minutes were plotted against the pH of the vessel. There was a rather sharp falling off on either side of the pH optimum which was, incidently, in accordance with the results obtained by other workers (Pappen-

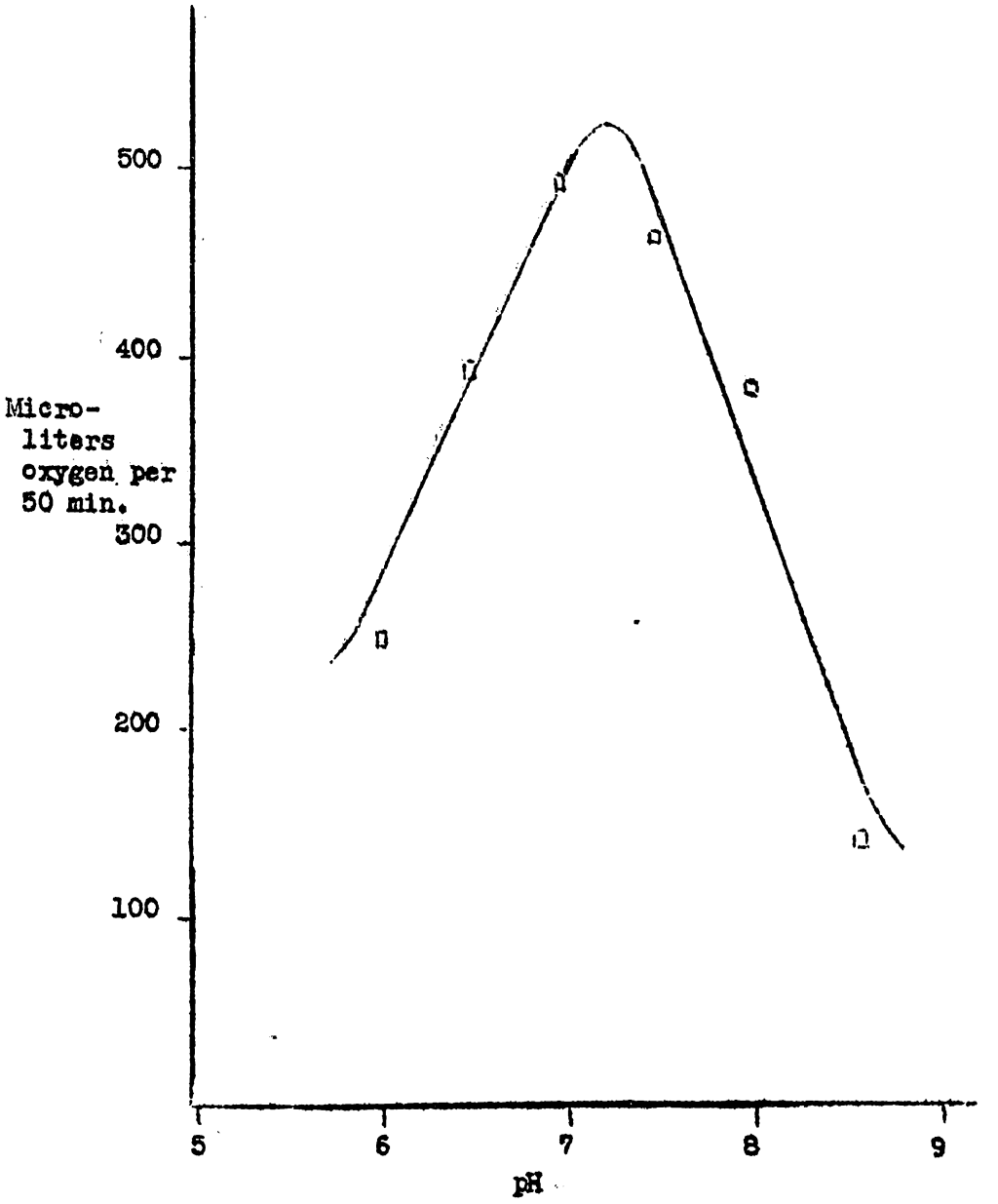

Fig. VI. Effect of pH on the Succinoxidase Activity in Sonic Extracts of Bacterium tularense. Each Warburg vessel contained 100 uM of succinate 1 ml. 0.1 M phosphate buffer, 1 ml. sonic extract. The final volume was 3 ml. The pH of the succinate and buffer were adjusted as indicated. The center well contained 0.2 ml. water. (Potassium hydroxide was not necessary since there is no carbon dioxide evolution during the course of these experiments.) The temperature was 37° C.

heimer (16), Kun (24), and Keilin (13)).

(2) The Effect of Substrate Concentration on Succinoxidase Activity

Figure VII shows the effect of increased concentration of succinate on activity. It may be seen from this figure that the rate rapidly falls off with time at low substrate concentration. In Figure VIII the substrate concentration was plotted against succinoxidase activity. The Michaelis constant was estimated from the graph as 7×10^{-3} M.

(3) The Effect of Cyanide and Sulfide on the Succinoxidase Activity of Bacterium tularense.

As may be seen in Table III both cyanide and sulfide inhibited the succinoxidase activity of Bacterium tularense. The curious aspect of this inhibition was that cysteine would completely reverse the inhibition produced by sulfide but did not reverse the inhibition produced by cyanide. This inhibition and reversal of inhibition was in keeping with the initial observation - that is, that during the growth of Bacterium tularense in casein decamin broth containing high amounts of cysteine, the growth of the organism was not inhibited even though large amounts of hydrogen sulfide were produced during the desulfhydration of this amino acid. Further, in experiments in which sulfide and cyanide were added to the broth, no inhibition was produced by the sulfide but complete inhibition was produced

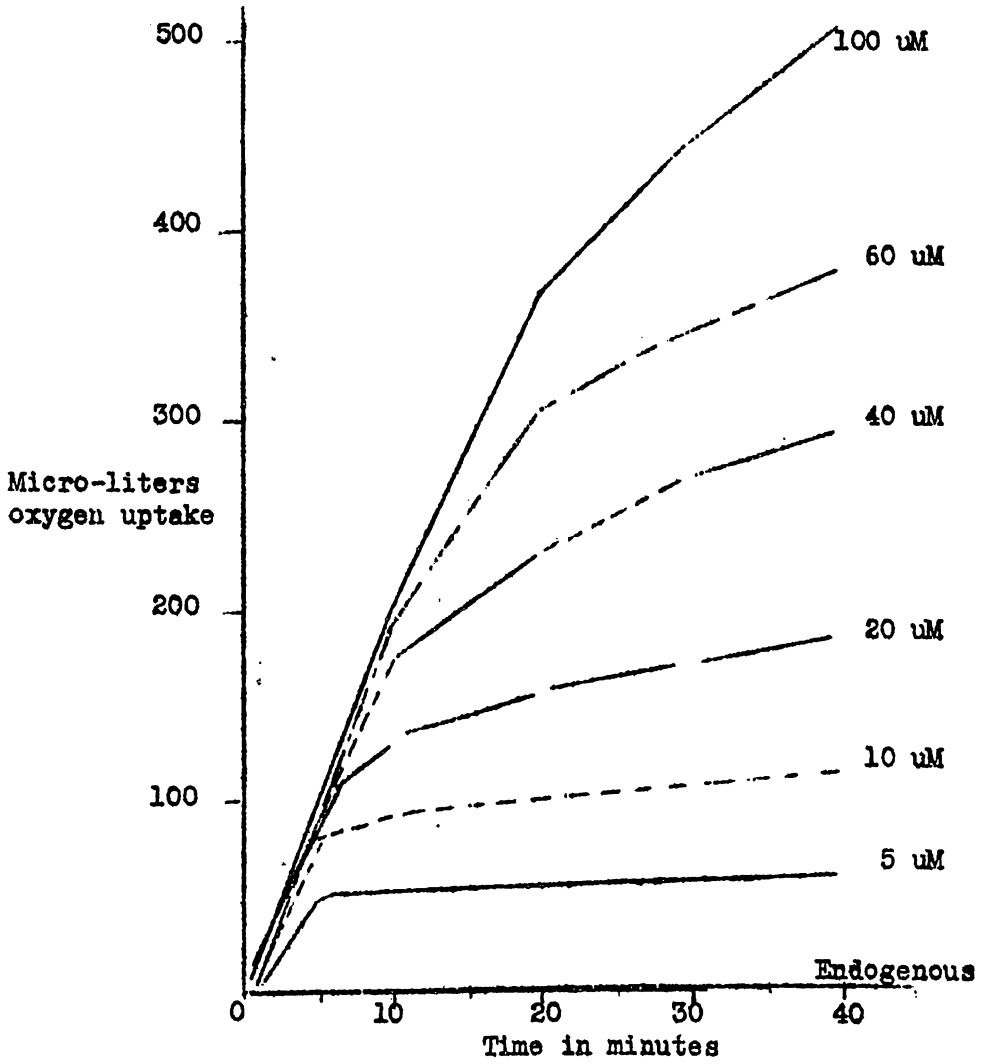

Fig. VII. The Effect of Substrate Concentration on the Rate of Oxygen Uptake by the Succinoxidase System of Bacterium tularense. The main compartment of the Warburg vessel contained 1 ml. of sonic extract, 1 ml. 01. M phosphate buffer pH 7.4, and 1 ml. of succinic acid pH 7.4 of the amounts indicated. The endogenous vessel contained all additions except substrate. The temperature was 37° C. The center well contained 0.2 ml. water.

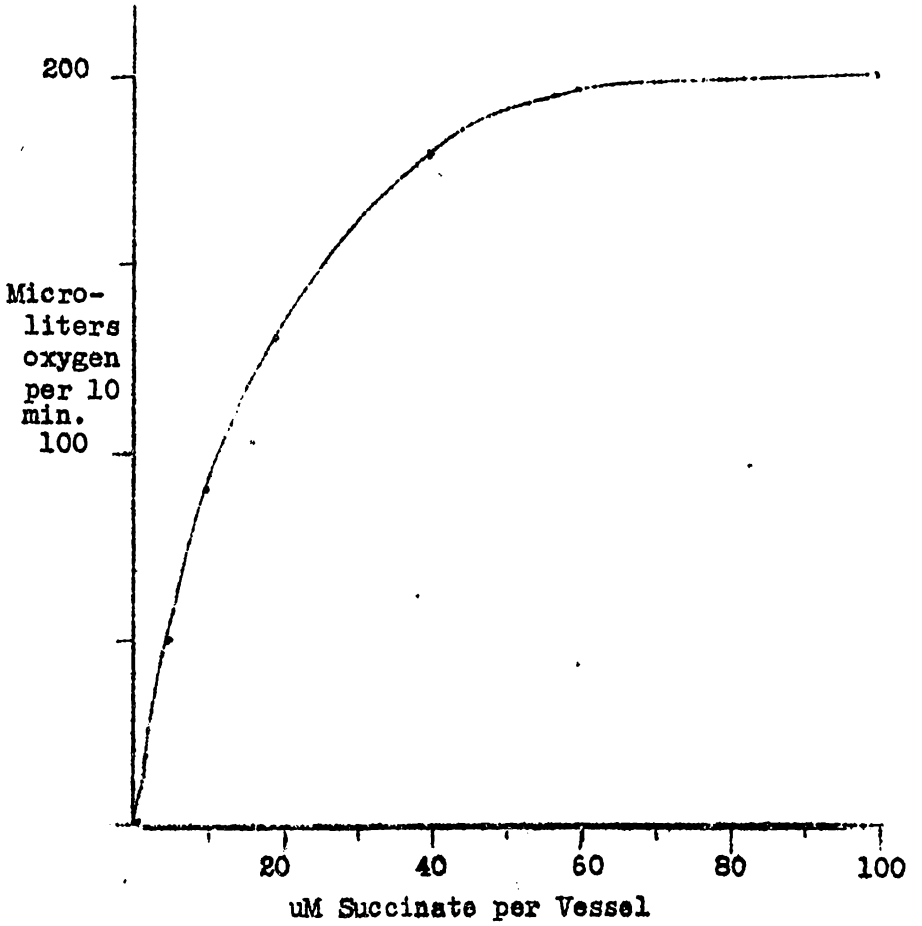

Fig. VIII. The Effect of Substrate Concentration on the Rate of Succinoxidase Activity in Sonic Extracts of Bacterium tularense. The experimental design is precisely as given in Fig. VII.

Table III

The Effect of Sulfide and Cyanide

on the Succinoxidase Activity of Bacterium tularense

	Component in Warburg Vessels	ul O_2/30 min.
A	0.5 ml. washed particles* / 2.5 ml. 0.1 M phosphate buffer	-
	Washed particles / 20 uM succinate / 1.5 ml. phosphate buffer	201
	Washed particles / 20 uM succinate / 2 x 10^{-3} M sulfide** / 1 ml. phosphate buffer	78
	Washed particles / 20 uM succinate / 2 x 10^{-3} M sulfide** / 10 uM cystiene / 0.5 ml. phosphate buffer	212
	Washed particles / 10 uM cystiene / 2.ml. phosphate buffer	-
	Washed particles / 10 uM cystiene / 2 x 10^{-3} M sulfide** / 1.5 ml. phosphate buffer	22
B		
	Washed particles / 2.5 ml. phosphate buffer	-
	Washed particles / 20 uM succinate / 1.5 ml. phosphate buffer	172
	Washed particles / 20 uM succinate / 1 x 10^{-3} M cyanide / 1 ml. phosphate buffer	104
	Washed particles / 20 uM succinate / 1 x 10^{-3} M cyanide / 10 uM cystiene / 0.5 ml. phosphate buffer	129
	Washed particles / 10 uM cystiene / 2 ml. phosphate buffer	-
	Washed particles / 10 uM cystiene / 20 uM succinate / 0.5 ml. phosphate buffer	212

* Washed particles. See Methods section.

** Sodium sulfide 2 x 10^{-3} M final concentration pH 7.4

/ Sodium cyanide 1 x 10^{-3} M final concentration pH 7.4

Each vessel contained 0.5 ml. "washed particles" preparation plus the addition indicated, which were prepared immediately before using. The final volume of the Warburg vessels was 3 ml. The temperature was 37° C., the pH 7.4; the center well contained 0.2 ml. water.

at 2×10^{-4} M cyanide. See Table IV. The role of cysteine in the growth of this organism is further emphasized by an experiment in which young organisms harvested at their rapid growth stage were washed and transferred to a medium containing no cysteine. These cells rapidly ceased to grow. See Figure IX.

(4) Measurement of Succinoxidase Activity of Bacterium tularense

By adding a small amount of sonic extract to a solution of succinate and following the increase in optical density at 240 mu. in a Beckman DU spectrophotometer, one is able to measure the rate of fumarate accumulation and thus the rate of succinic oxidase activity. From Figure X one may see that cyanide inhibits the succinoxidase activity of Bacterium tularense. The initial rate rapidly falls to zero in the cuvette containing cyanide in contrast to the cuvette containing no cyanide. Further from the slope of the line we may calculate succinoxidase activity in terms of uM per minute per ml. of sonic extract used. Thus from a standard curve of a plot of the optical density vs. concentration one may calculate that the rate of succinic acid oxidation is 6 uM per minute per ml. Also when one determines the succinoxidase activity of this preparation in a Warburg respirometer and then calculates the oxygen uptake in terms of fumaric acid produced one finds the rate to be 6.2 uM of fumarate per minute per ml. of enzyme. This

Table IV

The Effect of Sulfide and Cyanide

on the Growth of Bacterium tularense

Additions	Growth in Optical Density Units*
Basal medium**	0.569
Basal medium + 1000 gamma/ml. sulfide	0.553
Basal medium + 100 gamma/ml. sulfide	0.569
Basal medium + 10 gamma/ml. sulfide	0.553
Basal medium + 1 gamma/ml. sulfide	0.538
Basal medium + 1000 gamma/ml. cyanide	0.097
Basal medium + 100 gamma/ml. cyanide	0.097
Basal medium + 10 gamma/ml. cyanide	0.097
Basal medium + 1 gamma/ml. cyanide	0.420

* Determined after incubation for 16 hours at 37° C.

** Basal medium. (See Methods section). All additions were added to the media before autoclaving. Final volume in tubes 10 ml.; inoculated with 0.1 ml. of a 2 x washed suspension of Bacterium tularense having an OD of 0.482.

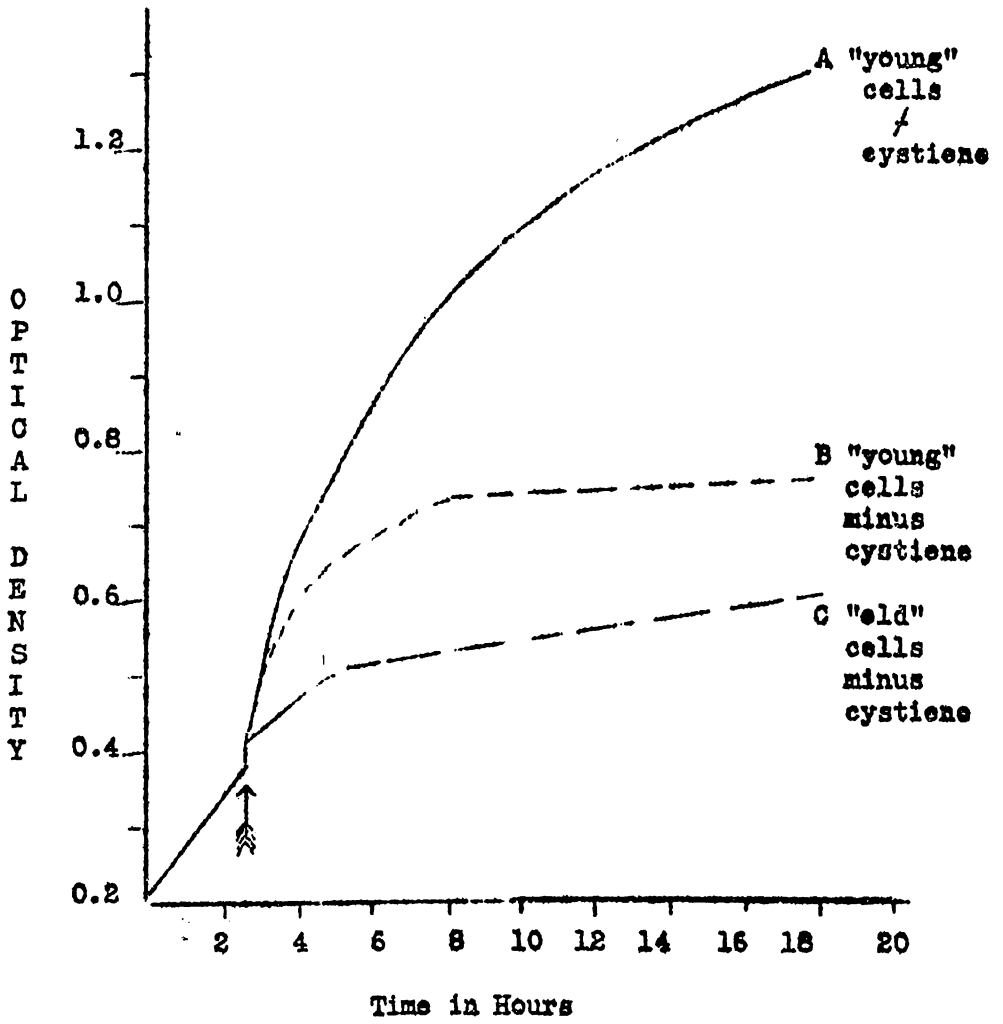

Fig. IX. The Effect of Cystiene on the Growth of Bacterium tularense. "Young" cells were grown on casein decamin media (described in Methods section). After 2 1/2 hours incubation the organisms were washed with 0.9% saline and placed (arrow) in A fresh casein decamin medium or B in casein decamin medium which contained no added cystiene. "Old" cells were obtained from a 24 hour culture, washed 2 times with 0.9% saline and C inoculated in casein decamin medium which contained no cystiene.

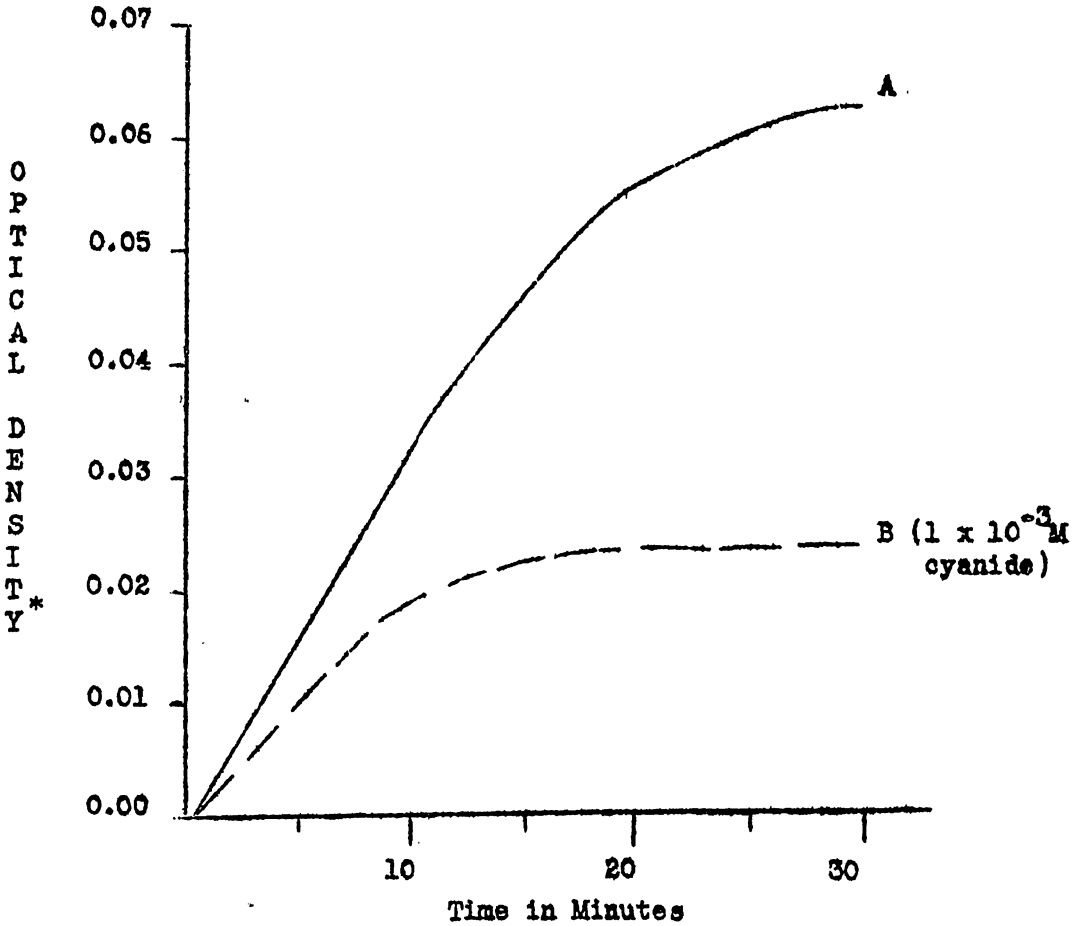

Fig. X. The Effect of Cyanide on the Succinoxidase Activity of Bacterium tularense. Cuvette A contained 1 ml. of succinate (100 uM) + 0.01 ml. sonic extract + 2.0 ml. 0.1 M phosphate buffer pH 7.4. Cuvette B contained 1 ml. of succinate (100 uM) + 0.01 ml. sonic extract + 0.5 ml. cyanide 1 x 10^{-3} M final concentration + 1.5 ml. 0.1 M phosphate buffer. The blank contained no substrate. Optical density was determined at 240 mu. with a Beckman DU spectrophotometer.

* Change in Optical Density.

slight increase in the rate over the rate of succinic acid oxidation determined in a spectrophotometer may be accounted for on the basis of temperature difference since the cuvette experiments were run around 30° C while the oxygen uptake determinations were run at 37° C.

Attempting to determine the succinic dehydrogenase activity using methylene blue in the Warburg respirometer showed that the methylene blue did not accelerate and in fact slightly inhibited the rate of succinoxidase activity. Further the inhibition of succinoxidase produced by cyanide was not reversed on the addition of methylene blue. This suggests that the cyanide inhibits at the cytochrome b step of succinic acid oxidation. See Figure XI. It should be pointed out that sonic extracts of Bacterium tularense are capable of reducing methylene blue in a Thunburg vessel and that this reduction may be reversed by the addition of fumaric acid. Further the reduced b band produced on the addition of succinic acid to sonic extracts of Bacterium tularense in a Thunburg vessel may be reduced in intensity and completely disappear on addition of methylene blue. Thus this would indicate that the methylene blue is capable of reoxidizing the cytochrome b and further that the rate of reoxidation of cytochrome b is not the rate limiting step since addition of methylene blue does not increase the succinoxidase activity.

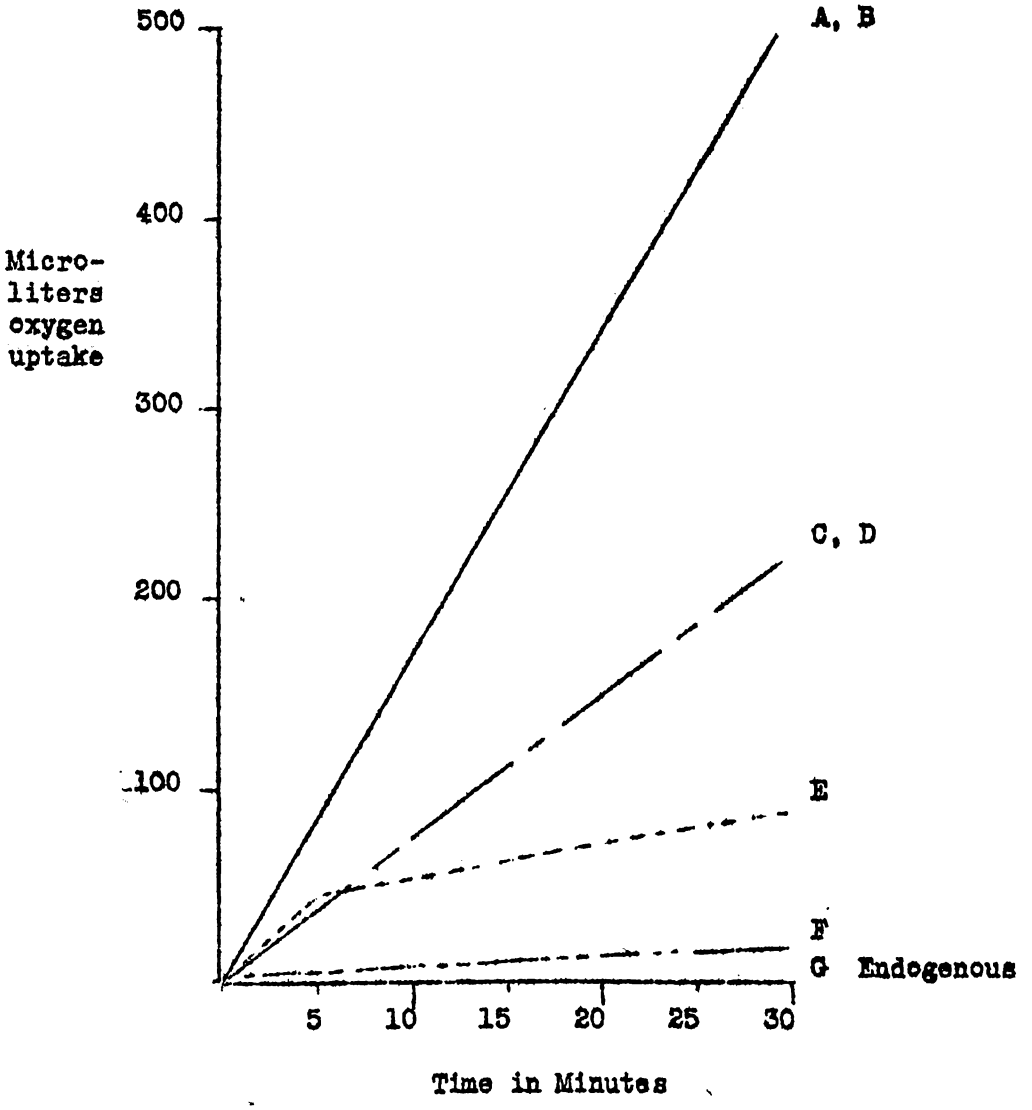

Fig. XI. The Effect of Enzyme Concentration and Methylene Blue on the Succinoxidase Activity of Sonic Extracts of Bacterium tularense in the Presence and Absence of Cyanide. Each Warburg flask contained 100 uM of succinate pH 7.4. In addition flask A contained 0.5 ml. sonic extract + 1.5 ml. of 0.1 M phosphate buffer; flask B contained 0.5 ml. sonic extract + 0.2 ml. 0.1% methylene blue + 1.3 ml. 0.1 M phosphate buffer; flask C contained 0.1 ml. sonic extract + 1.9 ml. 0.1 M phosphate buffer; flask D contained 0.1 ml. sonic extract + 0.2 ml. 0.1% methylene blue + 1.7 ml. 0.1 M phosphate buffer; flask E contained 0.5 ml. sonic extract + 0.5 ml. cyanide (1×10^{-3} M final concentration) + 0.2 ml. 0.1% methylene blue + 0.8 ml. 0.1 M phosphate buffer; flask F contained 0.1 ml. sonic extract + 0.5 ml. cyanide + 0.2 ml. methylene blue + 1.2 ml. 0.1 M phosphate buffer; flask G endogenous contained 0.5 ml. sonic extract + 2.5 ml. 0.1 M phosphate buffer with no succinate added. The temperature was 37° C. The center well contained 0.2 ml. water.

(5) The Effect of Cytochrome c on the Succinoxidase Activity in Sonic Extracts of Bacterium tularense

Keilin and Hartree (13) pointed out that small amounts of cytochrome c stimulated to a marked degree the activity of their succinoxidase preparation from heart muscle. The introductory discussion of this thesis includes work by these workers and others strongly implicating the role of cytochrome c in mammalian succinoxidase system. Therefore experiments were carried out to demonstrate whether or not cytochrome c plays a role in the succinoxidase system of Bacterium tularense. Table V shows the results of the addition of varying concentrations of cytochrome c on the succinoxidase activity of sonic extracts of Bacterium tularense. No stimulatory effects of cytochrome c could be demonstrated.

(6) The Effect of Hydrogen Peroxide on the Succinoxidase Activity of Bacterium tularense

It became apparent very early in our initial studies of the succinoxidase system of Bacterium tularense that the classical series of enzymes was not involved. Since the transfer of electrons did not involve a cytochrome oxidase with subsequent reoxidation by molecular oxygen to water, there remained the possibility that the reoxidation mechanism of cytochrome b by molecular oxygen yielded hydrogen peroxide rather than water as the end product. Experiments were carried out to attempt to demonstrate the role of

Table V

The Effect of Cytochrome c on the Succinoxidase Activity in Sonic Extracts of Bacterium tularense

Contents of Warburg Vessels	ul O_2/30 min.
Sonic Extract + 2.5 ml. 0.1 M phosphate buffer	-
Sonic Extract + 100 uM succinate + 1.5 ml. phosphate buffer	221
Sonic Extract + 100 uM succinate + 1×10^{-4} M cytochrome c* + 1 ml. phosphate buffer	230
Sonic Extract + 100 uM succinate + 5×10^{-5} M cytochrome c + 1 ml. phosphate buffer	224
Sonic Extract + 100 uM succinate + 1×10^{-5} M cytochrome c + 1 ml. phosphate buffer	238
Sonic Extract + 100 uM succinate + 5×10^{-6} M cytochrome c + 1 ml. phosphate buffer	246

* Cytochrome c solution was prepared immediately before use (obtained commercially).
Each vessel contained 0.5 ml. of sonic extract and the addition indicated to a final volume of 3 ml.; pH 7.4. The temperature was 37° C. The center well contained 0.2 ml. water.

hydrogen peroxide in the electron transport mechanism of the succinoxidase system. Since the organism and the sonic extracts of the organism were known to possess high catalase activity the initial attempt was that of coupling the possible hydrogen peroxide production with a peroxidative oxidation of another substrate catalyzed by catalase. This method has been successfully demonstrated by Keilin and Hartree (37). In this method the addition of ethyl alcohol to a preparation oxidizing a substrate with a concomitant production of hydrogen peroxide causes twice the expected oxygen uptake since the hydrogen peroxide produced is not broken down by the catalase present but rather is used to peroxidatively oxidize the ethyl alcohol. Further evidence is that acetaldehyde which is formed in this coupled oxidation may be found partially in the media and isolated as a derivative and partially in the center well containing potassium hydroxide. The potassium hydroxide in this case caused the base-catalyzed condensation of the acetaldehyde to polymeric products, which were brown in color.

Attempts made to demonstrate hydrogen peroxide production during the oxidation of succinic acid by sonic extracts or "washed particles" of Bacterium tularense were unsuccessful. Using either ethyl alcohol or a more easily oxidizable product such as cysteine did not affect the oxygen uptake during the oxidation of succinic acid by "washed particles" or sonic extracts of the organism.

Examination of the medium showed no acetaldehyde nor could any discoloration be seen in the center well of the Warburg vessel. See Table VI.

Reasoning that cyanide at low concentrations would inhibit the catalase present and not affect the succinic dehydrogenase, thereby allowing an accumulation of hydrogen peroxide, an attempt was made to demonstrate possible hydrogen peroxide production by inhibiting the catalase in the preparation. Table VI also shows that addition of small concentrations of cyanide did not affect the oxygen uptake during the oxidation of succinic acid.

Another attempt to demonstrate the possible production of hydrogen peroxide during the oxidation of succinic acid was made using a specific catalase inhibitor. In this case, ethyl hydrogen peroxide, synthesized as described in the methods section, was examined for its effects on the succinoxidase system. Rather than increasing the oxygen uptake of this system, there was marked inhibition of activity as shown in Figure XII. This inhibitory effect may be explained as being due to the transfer of electrons by the cytochrome b to the ethyl hydrogen peroxide instead of molecular oxygen. This explanation is in accord with the observation that the reduced band at 560 mu., obtained by the introduction of succinate to sonic extracts of Bacterium tularense in a Thunburg tube, could be completely oxidized by the addition of ethyl hydrogen peroxide.

Table VI

The Effect of Ethyl Alcohol and Different Levels of Cyanide on the Succinoxidase Activity of *Bacterium tularense*

Contents of Warburg Vessel	ul O_2/30 min.
Washed Particles + 2.5 ml. 0.1 M phosphate buffer	-
Washed Particles + 20 uM succinate + 1.5 ml. phosphate buffer	174
Washed Particles + 100 uM Ethyl Alcohol[1] + 1.5 ml. phosphate buffer	-
Washed Particles + 20 uM succinate + 100 uM Ethyl Alcohol + 0.5 ml. phosphate buffer	179
Washed Particles + 20 uM succinate + 1×10^{-3} M cyanide[2] + 1 ml. phosphate buffer	127
Washed Particles + 20 uM succinate + 1×10^{-4} M cyanide + 1 ml. phosphate buffer	176
Washed Particles + 20 uM succinate + 1×10^{-5} M cyanide + 1 ml. phosphate buffer	177

1-2 Solutions were prepared fresh. Warburg vessels contained 0.5 ml. washed particles (See Methods section), and the additions indicated to a final volume of 3 ml.; pH 7.4. The temperature was 37° C. The center well contained 0.2 ml. water.

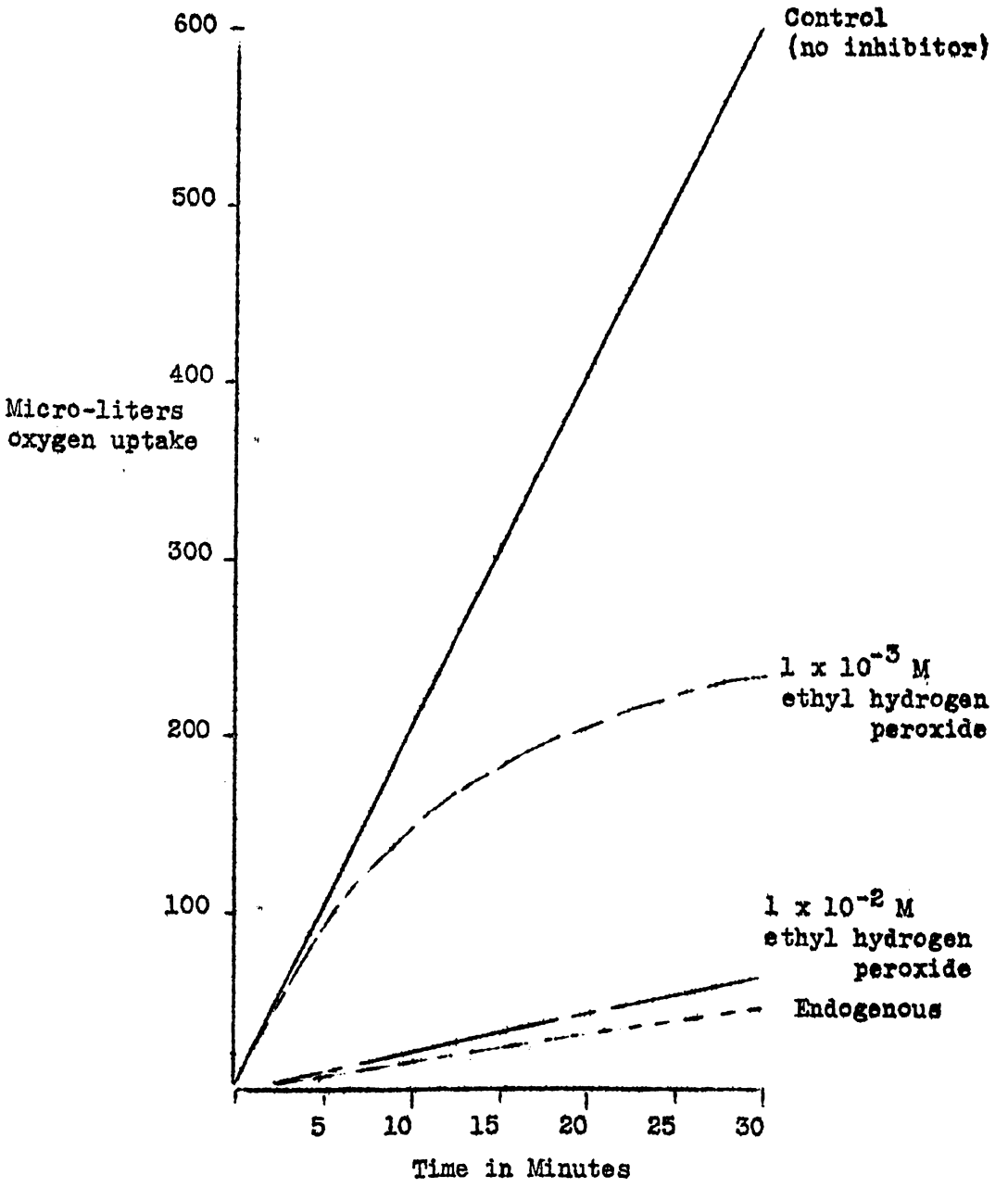

Fig. XII. The Effect of Ethyl Hydrogen Peroxide on the Succinoxidase Activity of Sonic Extracts of Bacterium tularense. Each Warburg vessel contained 1 ml. sonic extract + 100 uM succinate pH 7.4 + 0.5 ml. ethyl hydrogen peroxide to a final concentration of inhibitor as indicated in graph. The temperature was 37° C. The center well contained 0.2 ml. of water. The "endogenous" flask contained all additions except succinate.

The succinic dehydrogenase activity of Bacterium tularense extracts could be followed by the use of the dye 2-6-dichloroindophenol. When "washes particles" which were prepared as described in the Methods section were added to the dye containing succinate a fairly rapid reduction of the dye was evident. The addition of cyanide (5×10^{-4} M) to this causes a slight increase in the rate of reduction of the dye. On the other hand the addition of hydrogen peroxide (7×10^{-5} M) produces an inhibition of the reduction of the dye by these particles. Moreover, the addition of cyanide will reverse this inhibition by hydrogen peroxide. See Figure XIII.

(7) Evidence for a Thiol Group Functional in Succinic Dehydrogenase

If one treats a solution of the alcohol fraction of cytochrome b (See method.-page 62) with prolonged dialysis against distilled water or 0.001 M hydrochloric acid, it is observed that the succinic dehydrogenase activity and the succinoxidase activity was gone. Attempts to activate this preparation in the dialysate, with known co-enzymes such as DPN, TPN, riboflavin phosphate, riboflavin, and FAD were unsuccessful. However, as seen in Table VII addition of cysteine causes an almost complete return of activity. It would appear that dialysis allows a circulation of dissolved oxygen past sensitive thiol containing areas on the enzyme surface

Table VII

The Effect of Cystiene on the Oxidation of Succinic Acid by Treated Alcohol Fractions of Sonic Extracts of Bacterium tularense

Enzyme Treatment	Addition	ul O_2/30 min.
Dialysed Alcohol Fraction[1]	+ 2.5 ml. 0.1 M phosphate buffer	-
Dialysed Alcohol Fraction	+ 50 uM succinate + 1.5 ml. phosphate buffer	-
Dialysed Alcohol Fraction	+ 50 uM succinate + 10 uM cystiene + 0.5 ml. phosphate buffer	150
Untreated Alcohol Fraction[2]	+ 2.5 ml. phosphate buffer	-
Untreated Alcohol Fraction	+ 50 uM succinate + 1.5 ml. phosphate buffer	175
Untreated Alcohol Fraction	+ 50 uM succinate + 10 uM cystiene + 0.5 ml. phosphate buffer	204
Untreated Alcohol Fraction	+ 10 uM cystiene + 1.5 ml. phosphate buffer	-

1-2 See Text page 57. All solutions were prepared immediately before use. Each vessel contained 0.5 ml. of the fraction being tested (having 75 mg. dry wt/10 ml. of solution), plus the additions indicated. The final volume of the vessels was 3 ml.; pH 7,4. The temperature was 37° C. The center well contained 0.2 ml. water.

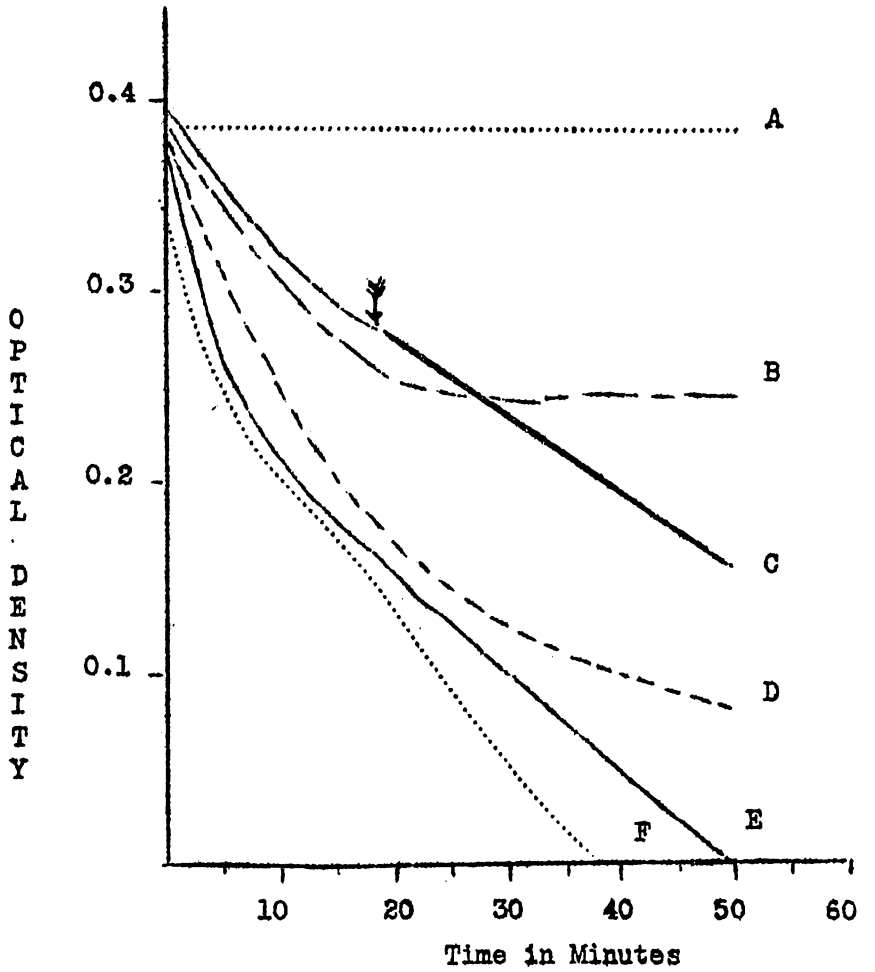

Fig. XIII. The Effect of Cyanide and Hydrogen Peroxide on the Succinic Dehydrogenase Activity of Bacterium tularense. Each tube contained 4.7 ml. of 1 mg/100 cc. solution of 2-6-dichloroindophenol + 0.1 ml. of "washed particles" + 1.0 ml. of 0.06 M succinate solution. A control tube A contained no substrate. Tube B contained all additions + 3.6×10^{-5} M (final concentration) hydrogen peroxide. Tube C was identical to B but at arrow 3.3×10^{-4} M cyanide (final concentration) was added. Tube D contained all additions + 3.3×10^{-4} M cyanide + 3.6×10^{-5} M hydrogen peroxide. Tube F, the control, contained all additions. Tube E contained all additions + 3.3×10^{-4} M cyanide. Water was used as a blank. The final volume was 6 ml., the pH 7.4. Optical density readings were determined with a Coleman Jr. spectrophotometer, at 600 mu. The temperature was 26° C.
At arrow 3.3×10^{-4} M cyanide (final concentration) was added.

bringing about the oxidation of the thiol groups with the resultant loss of activity.

H. The Absence of the Slater Factor in Sonic Extracts of Bacterium tularense

As mentioned in the Introduction, Slater (29) demonstrated a factor in mammalian succinoxidase which was inhibited by a variety of reducing agents, among which were cysteine, ascorbic acid, glutathione and more prominently British Anti Lewisite. Slater pointed out that the probable mechanism for this inhibition was in the peroxidative oxidation of the factor by hydrogen peroxide produced by the autooxidation of the previously mentioned reducing agents. Efforts were made to demonstrate this factor in sonic extracts of Bacterium tularense by the use of these reducing agents. Both ascorbic acid and cysteine were tried and both substances had no effect on the succinoxidase activity of this organism. See Table VIII.

Potter (38) using small concentrations of Antimycin has been able to demonstrate the inhibition of this so-called Slater factor by this substance. Small concentrations (1.5 gamma to 5 gamma) of Antimycin had no effect on either succinic dehydrogenase or succinoxidase activity of sonic extracts of Bacterium tularense. (39).

These experiments indicate that the Slater factor, which is part of the succinoxidase system in mammalian tissue is absent

Table VIII

The Effects of Cystiene and Ascorbic Acid

on the Succinoxidase Activity of Bacterium tularense Extracts

Contents of the Vessel		ul O_2/30 min.
Sonic Extract	+ 2.5 ml. 0.1 M phosphate buffer	6
Sonic Extract	+ 30 uM succinic acid + 1.5 ml. phosphate buffer	187
Sonic Extract	+ 30 uM succinic acid + 10 uM cystiene[1] + 0.5 ml. phosphate buffer	186
Sonic Extract	+ 30 uM succinic acid + 10 uM ascorbic acid[2] + 0.5 ml. phosphate buffer	188
Sonic Extract	+ 10 uM cystiene + 1.5 ml. phosphate buffer	19
Sonic Extract	+ 10 uM ascorbic acid + 1.5 ml. phosphate buffer	16

1-2 All solutions were prepared immediately before use. The vessel contained 0.5 ml. of Sonic Extract and the additions indicated. The final fluid volume of the vessels was 3 ml.; pH 7.4. The temperature was 37° C. The center well contained 0.2 ml. water.

in the succinoxidase system of Bacterium tularense.

I. Attempts to Isolate Succinic Dehydrogenase and Cytochrome b

Reference has already been made to the relationship of succinic dehydrogenase to cytochrome b in this organism. It has been pointed out that the succinoxidase activity was associated with the same material in fractional centrifugation as cytochrome b. Therefore, efforts were applied toward the separation and isolation of succinic dehydrogenase and cytochrome b. The cytochrome b was determined by examination of the fractions with a hand spectroscope. The succinic dehydrogenase activity was determined by the capacity of the fractions to reduce 2-6-dichloroindophenol in the presence of succinic acid. In some cases the succinoxidase activity of the fractions were determined in the Warburg respirometer.

(1) Alcohol Fractionation

50 ml. of the sonic extract were dialyzed overnight against one liter of distilled water at a temperature of 4° C. The sonic extract thus obtained was adjusted to pH 5.4 with acetate buffer. The extract appeared opaque immediately after the pH was adjusted and was therefore centrifuged. The supernatent and the precipitate thus obtained were separated and examined for cytochrome b and succinic dehydrogenase. The supernatent contained neither cytochrome b nor succinic dehydrogenase activity. The precipitate contained both.

This precipitate was resuspended in water and the solution adjusted to pH 6.5 with bicarbonate buffer. The tube containing the precipitate was then kept in a refrigerated bath at -5° C. while ethyl alcohol which had been previously cooled to -5° C. was added slowly with vigorous stirring until a concentration of 20% v/v ethyl alcohol was reached. The temperature was not allowed to rise over -2° C. during the addition of the alcohol and was at most times -5° C. After treatment with the alcohol the solution became opaque and was centrifuged in an International refrigerated centrifuge at a temperature of -5° C. The precipitate and supernatent were separated, diluted with water and lyophilized. At no time during the treatment with the ethyl alcohol was the temperature allowed to rise above -2° C. Both the supernatent and precipitate obtained by this latter treatment were examined for cytochrome b and succinic dehydrogenase activity. The supernatent contained neither cytochrome b nor succinic dehydrogenase, while the precipitate once again contained both. The precipitate was also examined for succinoxidase activity and found to be active. This precipitate could be dispersed in water or phosphate buffer but could be easily centrifuged from solution. An attempt was made to solubilize the precipitate by using 0.5% cholic acid pH 7.4. This treatment did not effectively solubilize or disperse the precipitate and when examined for activity it was found that

the cholic acid treatment had almost completely destroyed the succinoxidase activity. Attempts were made using chloroform to disperse the material and in this case also activity was completely destroyed.

(2) Treatment of Sonic Extracts with Various Materials

When sonic extracts of Bacterium tularense are treated with neutralized sodium desoxycholate to a final concentration of 2% the solution immediately cleared. In a typical experiment 0.8 ml. of sonic extract was mixed with 0.2 ml. of 10% desoxycholate. The solution cleared and was centrifuged for 30 minutes in an International high speed head centrifuge (25,000 x g). Only a very small precipitate was apparent on the bottom of the tube after centrifugation. The supernatent, which was clear, was examined for cytochrome b, succinic dehydrogenase and succinoxidase activity. On reduction with sodium hydrosulfite of the supernatent an intense band at 560 mu. was apparent. Thus the supernatent contained cytochrome b. However, when this material was examined for succinic dehydrogenase or succinoxidase activity it was shown to be devoid of any such activity.

On the assumption that the particles containing the succinic dehydrogenase and cytochrome b are cemented together and remain intact due to some lipid-like substance a second fat solvent was tried. In this case, 1 ml. of sonic extract was added to 1 ml. of

chloroform and slowly shaken to prevent foam and thus surface denaturation. The solution soon became milky in appearance and was centrifuged at 3000 x g in a Serval anglehead centrifuge. Examination of the tube showed that chloroform had collected on the bottom of the tube and was overlaid first by a white integument with a clear solution immediately above this. The white precipitate appeared to be denatured protein formed at the interface. The overlying supernatent was examined for cytochrome b and succinic dehydrogenase. It contained cytochrome b when examined with a hand spectroscope but showed no succinic dehydrogenase activity.

It has been recently demonstrated (40) that treatment of sonic extracts with protamine sulfate causes the extract to become turbid. After allowing the solution to remain at ice box temperature for one hour the contents of the tubes were centrifuged and the precipitate and supernatent were separated. Examination of the supernatent for succinic dehydrogenase activity showed that the supernatent was active and further, it contained cytochrome b. This treatment did not effect complete solublization since some of the cytochrome b plus oxidase was present in the precipitate, but affords a useful approach toward separation of the cytochrome b and succinoxidase.

DISCUSSION AND CONCLUSIONS

The proposed, and to some extent, established succinoxidase system of mammalian tissue involves the interplay of at least the following enzymatic reactions: succinic acid —— succinic dehydrogenase —— cytochrome b —— Slater factor —— cytochrome c —— cytochrome a —— cytochrome a_3 —— oxygen. From the foregoing results one discerns a strikingly different pattern of enzymes which carry out the oxidation of succinic acid in Bacterium tularense. This organism activates the substrate succinic acid and transfers the electrons to oxygen in a series of steps which seem to involve no more than two enzymes and possibly only one. The bulk of the work presented in this thesis deals with the mechanism of this succinoxidase and its enzyme components.

That the succinoxidase system found in Bacterium tularense is different from that found in mammalian tissue is immediately obvious from the experiments which show the lack of cytochrome oxidase in this bacterial cell. By two distinct methods - one involving the oxidation of p-phenylenediamine and measuring its oxidation whether by conventional Warburg technique or by the production of a violet color of the substrate, and the other by the attempted reoxidation of reduced cytochrome c, cytochrome oxidase has been shown to be absent in this organism. Further, the

absence of an absorption band in the red region of the spectrum associated with the presence of cytochrome oxidase also strengthens this argument.

That cytochrome c is not functional in the bacterial succinoxidase system is supported by the inability to extract cytochrome c from the cells, by the inability of the cells or cell extracts to reoxidize reduced cytochrome c, by the absence of an absorption band characteristic of cytochrome c in either the Soret region or at 550 mu., and finally by the failure of the addition of cytochrome c to stimulate the succinoxidase activity in the bacterial preparations. Also, a factor similar to the Slater factor appears to be absent.

As has been pointed out in the Results section of this thesis, cysteine, ascorbic acid, and antimycin A all fail to inhibit the succinoxidase activity in Bacterium tularense. Thus one may conclude on the basis of the foregoing that Bacterium tularense does not contain enzymes as part of its succinoxidase system which are functional beyond cytochrome b, and further that the dissimilarities extant between these bacterial preparations and mammalian tissue preparations lie in the absence of certain enzymes rather than the presence of different ones.

On the other hand, there are certain similarities which it may be worth while to point out. Most striking among these is the

presence of cytochrome b in this microorganism and its relationship to succinoxidase. The results reported in this thesis support the view that cytochrome b is identical to succinoxidase, but do not prove it. It has been shown that when sonic extracts of bacteria are treated with succinic acid under anaerobic conditions an absorption band appears at 560 mu., whose intensity may be reduced when oxygen is admitted to the system. Thus on the basis of this alone, one sees that the substrate succinic acid is activated by the sonic extract and may transfer its electrons to cytochrome b.

The relationship of succinoxidase to cytochrome b is further substantiated by the results obtained in attempts to isolate the cytochrome b from the succinoxidase. The data in Table II best shows the physical association of cytochrome b and succinoxidase. In this case the isolation procedures were carried out using the ultracentrifuge. One sees a roughly quantitative distribution of cytochrome b concentration and succinoxidase activity. All attempts to separate the cytochrome b from the succinoxidase were unsuccessful.

Other data supportive of the contention that cytochrome b is identical to succinoxidase have also been brought forth. Thus the thermolability of cytochrome b parallels the thermolability of the succinoxidase. Further the inhibitory effects of cyanide on succinoxidase and succinic dehydrogenase and the possible compound

formation of cyanide with cytochrome b strongly suggest the identity of the succinic dehydrogenase with cytochrome b. The addition of 1×10^{-3} M cyanide to Warburg vessels completely inhibits the succinoxidase activity; this inhibition cannot be reversed by methylene blue. This data indicates that the cyanide combines with some enzyme whose O-R potential is lower than methylene blue. In mammalian tissue it is easily observed that the inhibition produced by cyanide may be reversed by methylene blue. This reversal has been demonstrated as being due to the fact that the cyanide combines with the cytochrome oxidase and when one adds methylene blue, the inhibited system transfers the electrons to the methylene blue, thus allowing the oxidation of succinic acid to continue. Since our enzyme system does not contain a cytochrome oxidase it must contain an oxidase whose O-R potential is lower than methylene blue. This would explain why the dye does not reverse the inhibition.

The same general picture is observed when one determines the succinic dehydrogenase activity using ferricyanide as the electron acceptor. Once again cyanide inhibits the succinic dehydrogenase at the same concentrations necessary to inhibit the succinoxidase.

In the instance where the reduction of a dye is used as a measure of the dehydrogenase activity one does not see any effect of cyanide on the oxidation of succinic acid unless one pre-treats

the enzyme with cyanide. The explanation offered for this is simply that the amount of dye allowable in this system is necessarily small because of its inherent toxcity to enzyme systems. This small amount of dye is so rapidly reduced by the system that one may not see any effect on the activity by the addition of cyanide.

The results obtained from attempts to demonstrate a compound formation by cyanide with cytochrome b have been presented in the Results section. It appears that cyanide forms a compound with the oxidized form of cytochrome b. This spectrum obtained on the addition of cyanide to sonic extracts of the organism appears similar to the reduced absorption spectrum. One could expect that the porphyrin component of cytochrome b would react with cyanide.

One may conclude from this that the mechanism of inhibition of succinoxidase by cyanide involves a combination with the oxidized form of cytochrome b. This oxidized form seems to be in part operative as the succinic dehydrogenase. Since we have reported evidence that the succinic dehydrogenase contains a thiol group functional in carrying out the oxidation of succinic acid, we may conjecture an enzyme which has an active center involving a free thiol group in juxtaposition with a porphyrin moiety. One could picture the succinic acid forming an activated complex with the succinic

dehydrogenase by combining at the "thiol center". This activated complex would then facilitate the transfer of electrons from the succinic acid to the porphyrin moiety and thus reduce the iron from ferric to ferrous. The ferrous iron is oxidized by molecular oxygen which combines with the protons formed during the oxidation of succinic acid to produce a molecule of water in the manner shown in Figure XIV. This general scheme is consistent with the results obtained in this study.

It will be remembered that the work pursued in this thesis was initiated by the questions raised concerning the effects of hydrogen sulfide produced from the desulfhydration of cysteine on the electron transfer mechanism in the organism. Our results have shown that hydrogen sulfide is indeed inhibitory to the succinic dehydrogenase but that this inhibition may be reversed by cysteine. Thus the very substrate from which an inhibitory substance is formed, itself reversed the effects of the inhibitory substance. This would explain why high cysteine concentrations are necessary throughout the growth of the organism, (See Figure IX) but it throws no light on the function of cysteine in the metabolism of this microorganism.

It should be reported here in passing that some consideration has been given to the question as to what effect hydrogen sulfide has on other organisms. In this regard, the microorganism Bacillus subtilis was selected since this organism possesses a complete

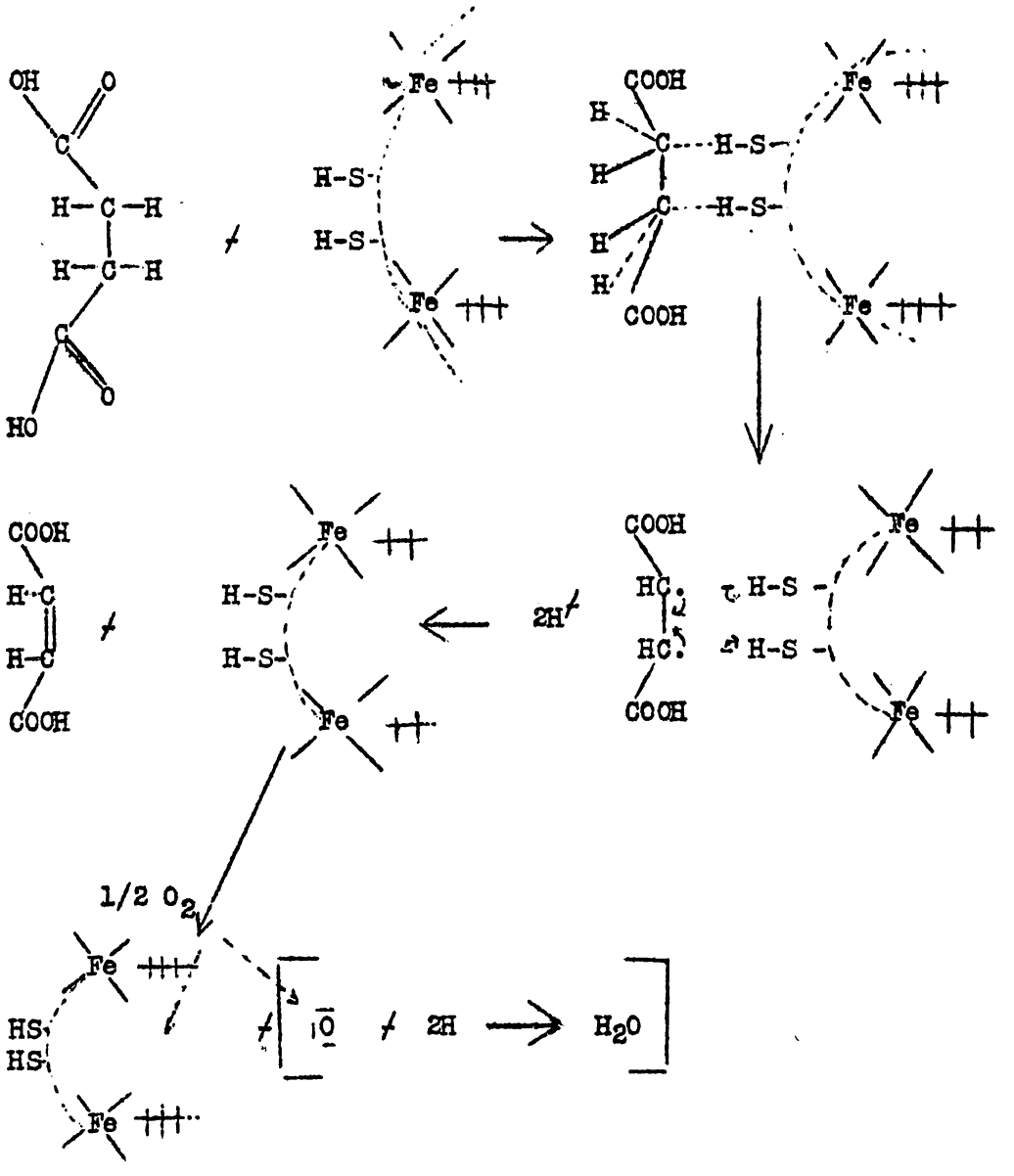

Fig. XIV. Model of Succinoxidase System in Bacterium tularense

cytochrome spectrum and has been reported to produce hydrogen sulfide from various substrate sources. The organism was grown on nutrient agar and on examination found to contain cytochromes a, b, and c. In one experiment two groups of organisms were grown on nutrient agar. In one group 0.1% sodium sulfide was added to the nutrient agar before autoclaving. The flasks of agar were inoculated with an inoculum from an 8 hour broth culture prepared from a fresh agar slant. After 24 hours incubation at 37° C. the bacteria were collected from the agar surface, washed with saline and the reduced spectrum was examined with a hand spectroscope. The normally grown organisms showed a typical 3-banded spectra (d = 530 mu; c - b = 548 mu. - 562 mu.; a = 595 mu. - 604 mu.). The organisms grown in the presence of hydrogen sulfide showed only a b' band (b' = 551 - 559 mu.). Also, while the normally grown organisms showed active cytochrome oxidase (determined with the use of the Nadi reagent) the organisms grown on sulfide agar were inactive. These experiments indicate that the cytochrome system of a microorganism is a relatively labile one whose components may change on the appearance of an alteration in the environment which would tend to prevent normal functionings of this system.

It is interesting to conjecture as to whether the Bacterium tularense organism contains a single cytochrome component because

of its special growth requirements which demand high concentrations of cysteine, thus producing environmental conditions that necessitate an alternate electron transfer mechanism.

SUMMARY

The succinoxidase system in sonic extracts of Bacterium tularense has been investigated. It has been shown that this microorganism does not contain cytochrome oxidase, cytochrome a, cytochrome c or a "Slater" factor in contrast to mammalian tissue. Evidence has been brought forth that supports the view that the succinoxidase system in this microorganism consists of succinic dehydrogenase and cytochrome b. The latter acts as an oxidase in the system.

Some properties of the succinoxidase system have been explored. Optimum activity occurs at pH 7.3. The Michaelis constant was determined as 7×10^{-3} M. The effect of certain inhibitors on the activity of the system was examined. The succinic dehydrogenase was demonstrated to be a thiol-enzyme.

The properties of the cytochrome b component of the system were examined. The cytochrome showed absorption bands at 430 mu, 525 mu, and 560 mu. The O-R potential was estimated as approximately 0.04 volts. It is thermolabile.

The relationship of cytochrome b to succinic dehydrogenase was examined. The results support the view that the two enzymes may be identical.

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